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THE INFLUENCE OF MOISTURE ON THE EGGS OF *AUSTROICETES CRUCIATA* SAUSS. (ORTHOPTERA) WITH REFERENCE TO THEIR ABILITY TO SURVIVE DESICCATION

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(From the Waite Agricultural Research Institute).

(Accepted for publication 26th January, 1942.)

In South Australia the eggs of *Austroicetes cruciata* are laid in November and hatch during the first half of September; they remain in the soil for about ten months. During this time the eggs normally pass through a hot, dry summer and in some years the water content of the soil around them may be below the wilting point for periods in the winter and spring. Thus the eggs may be subject to desiccation at various stages of their development. They are in a state of diapause during the summer and until about the end of May (Birch, 1941). In this paper we report experiments concerning the ability of the eggs of *A. cruciata* to survive desiccation at different stages of development.

Little is known about the influence of the stage of development of the embryo on the ability of insect eggs to survive desiccation. Thompson and Bodine (1936) found that the post-diapause eggs of *Melanoplus differentialis* lost water more rapidly than eggs in diapause. The reasons for such differences are not yet clear, but in the case of grasshopper eggs it is important to consider the egg membranes. At the time of oviposition only the vitelline membrane and chorion are present; the cuticle is secreted later; it consists of two layers, a thin yellow layer and a thicker white layer; the latter disintegrates before the nymph hatches (Slifer, 1937). Eggs of *A. cruciata* collected from the field three days after they had been laid were sectioned; at this stage neither the white nor yellow cuticle had been secreted. In eggs incubated at 22·5° C. the white cuticle began to disintegrate 3–4 days before hatching. No morphological changes in the membranes were detected at any other stage of development of the embryo, so that apparently some other factors are responsible for the changes in the ability of the egg to survive desiccation.

The eggs of *A. cruciata*, like those of other Acrididae, absorb water during their development (Bodine, 1929; Roonwal, 1936).

The Absorption of Water.

Longitudinal sections show that the eggs of *Austroicetes cruciata* possess a "hydropyle area" at the posterior pole similar to that described by Slifer (1938) for *Melanoplus differentialis*. It is through this area that water is absorbed.

The water content of eggs which had absorbed the maximum amount of water was determined at various stages of development. The figures for eggs in diapause were obtained from eggs collected in the field at regular intervals between March 11th and May 16th, 1940. The egg pods were kept moist for 24 hours before they were dissected, the individual eggs then being placed on moist blotting paper for a further 24 hours at 13·5° C., to allow them to absorb the maximum amount of water. They were then placed on dry blotting paper for about one minute until the chorion had become opaque, indicating surface dryness. Duplicate lots of 75 eggs were dried at 105° C.; the water content was given as a ratio *loss of weight* to *weight of dry matter*. The figures for post-diapause eggs were obtained from eggs collected on April 30th and placed at 13·5° C. for 50 days and 8° C. for 7 days to eliminate diapause. These eggs were incubated on moist plaster of Paris at 22·5° C. and weighed daily after drying on blotting paper (as described above). The results are shown in Table 1. The gradual disintegration of the white cuticle a few days before hatching was probably responsible for the erratic results at this stage.

The mean weight of dry matter per egg decreased from 1·57 mg. for eggs collected in March to 1·48 mg. for eggs collected in July; the weight of dry matter in eggs collected on September 2nd, 1939, was 1·26 mg.

TABLE 1.

Showing the increase in water content of eggs during development.

Date collected.	Water content. Dry matter.	Net weight (gm.) 100 eggs.	Stage of development.	
11th March	2·13	0·492	III*–VI	Diapause
16th April	2·30	0·539	VIII–IX	
30th April	2·28	0·517	IX	
16th May	2·96	0·586	X None hatched at 30° C.	
29th May	3·14	0·657	X–XIII 66 p.c. hatched at 30° C.	
Days at 22·5° C.				
0	—	0·659	Eyespots of eggs at posterior poles	Post-diapause
1	—	0·664	Eyespots in central position. Fluid accumulated at posterior pole	
2	—	0·676	Eyespots within ¼ length of egg from anterior pole	
3	—	0·689	Eyespots at anterior pole	
4	—	0·700		
6	—	0·711		
7	—	0·713	Yolk engulfment complete	
8	—	0·716		
9	—	0·714		
10	—	0·716		
11	—	0·715	White cuticle thinner	
13	—	0·720		
14	—	—	First egg hatched. White cuticle disintegrated. The mode of hatching curve occurred on the 15th day	

* The roman numerals refer to stages of the embryo described by Steele (1941).

The rate of absorption of water by eggs in diapause was determined. Four lots of 100 eggs which were absorbing water from moist blotting paper at 25° C. were weighed at 5-hourly intervals until equilibrium had been reached. They had previously been dried out until the 400 eggs weighed 1·200 gm. The eggs become turgid in 48 hours and reached equilibrium in about 70 hours. The increases in weight are shown in Table 2.

TABLE 2.

Showing the rate of absorption of water by eggs in diapause at 25° C.

Hours.	Increase in weight (gm.) 100 eggs.	Hours.	Increase in weight (gm.) 100 eggs.
0	0·000	40	0·125
5	0·024	45	0·128
10	0·045	50	0·134
15	0·067	55	0·134
20	0·084	60	0·136
25	0·089	65	0·143
30	0·098	70	0·145
35	0·104	75	0·145

The Rate of Loss of Water.

The rate of loss of water was determined for eggs in nine different stages of development. The eggs used in this experiment were collected at Wilmington between November, 1939, and July, 1940. Newly-laid eggs were collected on November 11th and placed at 12° C. in an ice chest immediately they were removed from the egg bed; they remained at this temperature for 6 days and were then placed at 13·5° C. in the laboratory for 10 days before being dissected from the pod. Some eggs collected on November 11th were kept in the laboratory at room temperature

until December 23rd; the stage of development of these eggs on that date was considered to correspond to that of eggs in the field on December 23rd. The data for the remaining seven stages of development were obtained from eggs collected at regular intervals between March and July, 1940.

The egg pods were moistened to facilitate dissection, the separated eggs then being placed on moist blotting paper for 48 hours at 13·5° C. until they had become turgid. Before weighing, they were placed on dry blotting paper for about one minute until the surfaces of the eggs were quite dry. The eggs were then exposed in air at relative humidities of 22 p.c. (s.d. = 0·53 inches of mercury) and 55 p.c. (s.d. = 0·31 inches of mercury) at 20° C.; they were weighed daily for some weeks and then at less frequent intervals until about one milligram of water remained per egg. Three replicates of 80 eggs were used for each stage of development and each lot weighed to the nearest half milligram. The humidities were maintained in Fowler jars which contained the appropriate concentration of sulphuric acid (National Research Council, 1926).

The five curves shown in Fig. 1 illustrate the rate of loss of water at 55 p.c. R.H. for eggs collected in the field on the five dates indicated: on each of these dates the eggs were in different stages of development (Birch, 1942). For each curve the weight of 100 eggs was plotted against time.

In comparing the curves for successive stages of development of the egg, use has been made of orthogonal polynomials (Fisher and Yates, 1938). Instead of fitting the polynomial regression $y = a + bx + cx^2$ the equivalent equation $y = A + B\xi_1 + C\xi_2$ is used. The number of points was reduced by taking means of successive triplicates. The values for B and C are shown in Table 3.

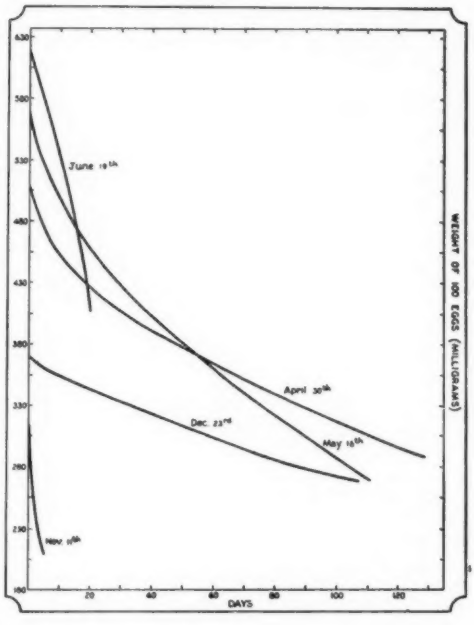

Fig. 1. Curves showing the rate of loss of water from eggs of *A. cruciata* at 55 p.c. R.H. (20° C.) collected from the field on the dates indicated.

TABLE 3.

Showing the values for B and C in the equation $y = A + B\xi_1 + C\xi_2$ for the curves of rate of loss of water from eggs at various stages of development.

Date collected from field.	55 p.c. R.H.		22 p.c. R.H.	
	B	C	B	C
11th November	— 0·04400	+ 0·006330	— 0·04400	+ 0·006330
23rd December	— 0·00046	+ 0·000035	— 0·00184	+ 0·000075
11th March	— 0·00391	+ 0·000463	— 0·00637	+ 0·000788
16th April	— 0·00181	+ 0·000029	— 0·00655	+ 0·000551
30th April	— 0·00164	+ 0·000160	— 0·00306	+ 0·000255
16th May	— 0·00279	+ 0·000204	— 0·00441	+ 0·000295
29th May	— 0·00897	+ 0·000633	— 0·01167	+ 0·002582
19th June	— 0·02343	— 0·001188	— 0·01770	+ 0·004757
18th July	— 0·02416	— 0·000714	— 0·02210	+ 0·000406

The factor B expresses the average slope of the curve; the factor C indicates the acceleration away from the average slope. Since B and C are correlated the value of C must be considered in relation to the corresponding value for B when reading the columns vertically.

In Fig. 2 the values of B for the dehydration curves given by eggs collected in the field between November, 1939 and July, 1940 are plotted against time. The steeper the dehydration curve the larger is B; thus in Fig. 1 the curve for eggs collected on June 19th has a larger B factor than the curve for eggs collected on December 23rd. As the rate of loss of water from the eggs increases, the slope of the dehydration curve increases so the value of the factor B is a measure of the egg's susceptibility to desiccation, and Fig. 2 traces the changes in this susceptibility during the period the eggs are developing in the field.

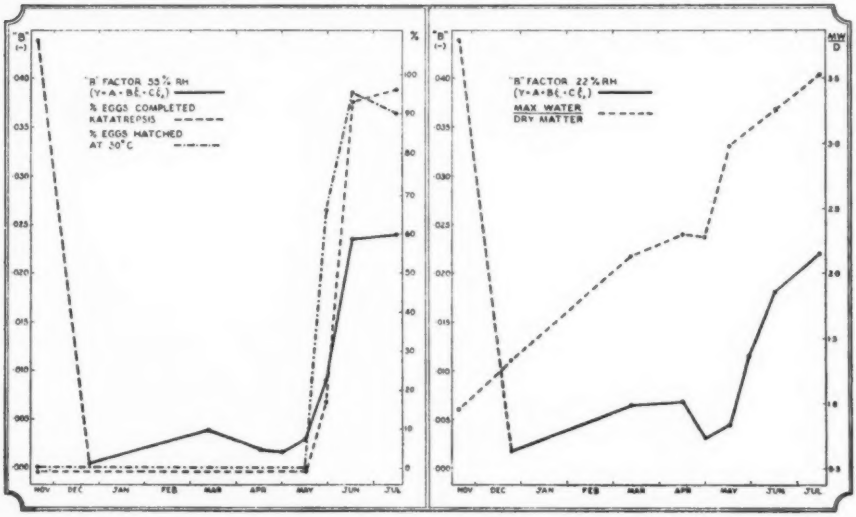

Fig. 2. The size of factor B indicates the susceptibility of eggs of *A. cruciata* to desiccation. The decrease in the spring (Nov., Dec.) is associated with the laying down of the cuticle; the increase in autumn (May) is associated with the elimination of diapause. The percentage of eggs to complete katatrepsis in the field and the percentage able to hatch at 30° C. indicate the extent to which diapause had been eliminated.

The Mortality Rate under Desiccation.

The mortality rate of the eggs under desiccation depends upon the stage of development of the embryo (Table 4).

TABLE 4.

Showing the influence of stage of development of the embryo on the mortality rate of eggs during desiccation.

Date collected. from field.	Stage of development.	Saturation deficit (inches of mercury) over conc. sulphuric acid.	Days dehydrated.	P.c. Mortality.
16th April	*VIII–IX (diapause)	0·68 (20° C.)	85	33
19th June	XIV (post-diapause)	0·45 (13·5° C.)	52	65
18th July	XV (post-diapause)	0·45 (13·5° C.)	20	40

* The roman numerals refer to stages of the embryo described by Steele (1941).

The minimum moisture content at which eggs were able to remain alive was determined. Post-diapause eggs were dried over concentrated sulphuric acid (at 13·5° C.) and at regular intervals batches of eggs were removed and incubated on moist plaster of Paris at 25° C.; a note was made of the number of eggs which failed to develop. In this way it was possible to determine the duration of exposure necessary to kill about 50 p.c. of the eggs. Batches of eggs which had been dried for the time necessary to kill about 50 p.c. were sorted, those which were turgid and those which were obviously dead being discarded. One hundred of the remaining eggs were

weighed separately to the nearest 0·02 mg., and then incubated at 23° C. on moist blotting paper. The eggs which failed to become turgid and develop were considered dead, the remainder were considered as living. All eggs, living and dead, were dried at 105° C. and the dry matter of the individual eggs recorded to the nearest 0·02 mg. Eggs at two stages of development were used, namely some collected on June 19th and some collected on July 18th. Because of the variability in the water content of the eggs which remained alive in these experiments, much larger numbers would be required for the comparison of eggs at different stages of development; hence in the following summary the results of the two stages have been combined: 40 p.c. of the eggs were alive after having been dried out as described, the ratio of water to dry matter in these living eggs varied from 0·5 to 2·8 as follows: 2 between 0·2–0·6, 10 between 0·6–1·0, 19 between 1·0–1·4, 17 between 1·4–1·8, 19 between 1·8–2·2, 13 between 2·2–2·8. The ratio of water to dry matter in the dead eggs varied from 0·2 to 2·6. The viable egg with the lowest water content had a *water content: dry weight ratio* of 0·5, i.e. it contained 0·65 mg. of water, having lost about 4·5 mg.

The mortality rate of eggs in diapause exposed to desiccation for periods from 1–7 months was determined. The eggs were collected at Wilmington between November 10th and 17th, 1939. The egg pods were moistened for several days during which the eggs became turgid; they were then dissected from the pods and stored at 23° C. and 75 p.c. R.H. for two weeks. After discarding the shrivelled eggs, the healthy turgid eggs were divided into 24 lots; eggs were exposed in desiccators for 30, 90, 150 and 210 days at 30° C., 35° C. and 40° C. and at relative humidities of 55 p.c. and 75 p.c. at each temperature. At the end of the treatment the eggs were incubated on moist plaster of Paris at 25° C.; after 7 days eggs which had failed to become turgid or had become mouldy were considered as dead. The mortality rate has been related to evaporation. The total evaporation is proportional to the product of the saturation deficit of the atmosphere and the period of exposure. In Fig. 3 the values along the abscissa are for saturation deficit in inches of mercury × time in days. The diagram shows the per cent. mortality rate with respect to evaporation and the duration of exposure; the temperatures at which the eggs were kept are also indicated in the figure. Each treatment was done with 5 replicates of 40 eggs. A difference in the mortality rate of 9·6 p.c. is significant at the 1 p.c. level of probability.

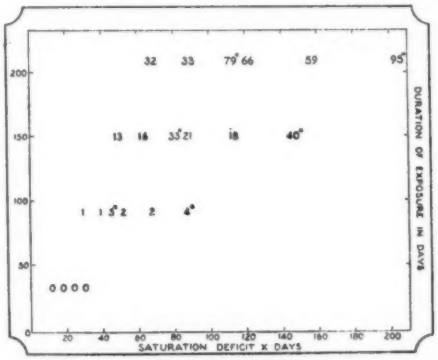

Fig. 3. Showing the mortality rate of eggs in diapause of *Austroicetes cruciata* under desiccation. The ordinates show the duration of the exposure; the abscissae indicate the severity of the desiccation since total evaporation is proportional to the product of saturation deficit and the duration of the exposure. Figures marked with an asterisk were obtained at 40° C., the others at either 30° C. or 35° C.

The Ability of Eggs to Develop and Hatch in the Absence of Free Water.

Eggs collected in the field at fortnightly intervals between May and July, 1940, were moistened until they became turgid. They were then separated from the pods and exposed at 18·9° C. to relative humidities of 22 p.c. and 55 p.c. The eggs in the controls were incubated on moist plaster of Paris. The numbers which hatched are shown in Table 5.

TABLE 5.

Showing the ability of eggs to hatch at 22 p.c. and 55 p.c. R.H. (18·9° C.).

	Relative humidity.				
	22 p.c.		55 p.c.		P.c. hatch
Date collected from field.	P.c. hatched.	Range of hatching (days).	P.c. hatched.	Range of hatching (days).	in control.
16th May	0	—	0·5	38	16
29th May	0	—	41·0	22–36	95
19th June	0	—	50·0	14–23	91
18th July	12·0	9–23	44·0	9–15	90

DISCUSSION.

There was little difference between the maximum amount of water absorbed by eggs at different stages of development during diapause; but a sudden increase in the water content of turgid eggs occurred at the end of the diapause period, i.e. about mid-May in the field. The water content of turgid eggs steadily increased from the end of diapause until hatching (Fig. 2). This trend in water content is similar to that in eggs of *Melanoplus differentialis* (Bodine, 1929).

Lillie (1916) has shown that when "K" in his empirical formula for the rate of absorption of water by aquatic eggs is constant, the process may be explained in terms of physical laws. The "K" values for the absorption of water by eggs of *A. cruciata* were constant.

The degree of susceptibility to desiccation of eggs at different stages of development, has been assessed in three ways: (1) in terms of the rate of loss of water at different humidities, (2) by the duration of the period of desiccation necessary to produce a given mortality, and (3) by the minimum water content necessary for life.

The rate at which eggs lost water when they were exposed to "dry" air depended on the stage of development of the embryo. It was greatest for newly-laid or prediapause eggs, relatively slow for diapause eggs (i.e. eggs collected in the field from December to mid-May); it became progressively greater for eggs collected after mid-May. When the weight of the drying eggs was plotted against time, the curves obtained with eggs collected after May 29th were not significantly different from straight lines; the curves for all preceding stages except pre-diapause showed an initial steep slope which gradually flattened out (Fig. 1). The linear nature of the curves for eggs in the post-diapause condition was evident from the small values for C relative to the corresponding values for B in Table 3. Eggs collected from mid-May onwards were able to develop slowly at 20° C. so that the embryos were at a more advanced stage at the end than at the beginning of the experiment. A slight concavity of the curves to the time axis (small negative values for C) evident in the curves for the last two stages at 55 p.c. R.H., is associated with the disintegration of the white cuticle before hatching. The curves for eggs collected in mid-May were intermediate between the two main groups of diapause and post-diapause eggs. The curves for eggs collected on March 11th and April 16th showed a slightly greater slope than for eggs collected on April 30th; this, however, is small compared to the change which occurred at the end of May. The shape of the curves for eggs in diapause are typical of the drying curves for insects in general. The size of the factor B indicates the susceptibility of the eggs to desiccation. In Fig. 2 the correlation between the ability of eggs to take in water, their susceptibility to desiccation and the breakdown of diapause is clearly indicated.

A similar change in the moisture relations of the egg occurs in *Melanoplus differentialis* (Thompson and Bodine, 1936). The difference between pre-diapause and diapause eggs in this respect is associated with the absence of both the yellow and the white cuticle in pre-diapause eggs. The difference between diapause and post-diapause eggs is not associated with any detectable change in the morphology of the egg membranes. The membranes of post-diapause eggs are considerably stretched by virtue of the greater water content of the eggs; Jahn (1936) has shown by measuring electrical capacitance, that the egg membranes in *M. differentialis* are thinner in post-diapause eggs. The amount of stretching in eggs of *M. differentialis* is about the same as in eggs of *A. cruciata*. However, the permeability of biological membranes is not merely dependent on morphological features but is also largely governed by the metabolic state of the lining protoplasmic layer. The property of semi-permeability of this layer is inseparable from the living condition, and is actually maintained by a constant process of metabolism (Lucké and McCutcheon, 1932). Dead eggs of *A. cruciata* do not become turgid when moistened. The change in permeability to water from the diapause to the post-diapause condition may thus be due to the increased metabolic rate. The increased permeability to the intake of water and the increased susceptibility to desiccation of sea urchin eggs when fertilized (Lillie, 1916, 1918), provides an interesting comparison with the similar change in grasshopper eggs as they pass from the diapause to the post-diapause condition. Bodine (1934) showed that the eggs of *Melanoplus* when in diapause resembled unfertilized sea urchin eggs in that the rate of oxygen consumption was not

reduced when the eggs were immersed in a 0·01 p.c. solution of HCN; whereas eggs in the pre-diapause and post-diapause stages resembled fertilized sea urchin eggs in that the same treatment caused a big, but reversible, reduction in the rate of oxygen consumption.

Eggs in the post-diapause state are more easily destroyed by desiccation than eggs in diapause (Table 4). It has not been possible to determine if the minimum amount of water necessary to keep the embryos alive varies from stage to stage, but it has been shown that some eggs in the post-diapause state can survive with "low" water contents, e.g. some eggs survived after the *water content*: *dry weight* ratio had been reduced from 3·2 to 0·6. Variability in rate of drying and susceptibility to desiccation varies greatly with individual eggs at the same stage of development. Similar variability has been found in the post-embryonic stages of other insects (Gunn, 1933).

When eggs in diapause were desiccated for periods from 1 to 7 months, the mortality rate was dependent on total evaporation and the duration of exposure. This is clearly indicated in Fig. 3 in which all the high figures for mortality are on the right hand side of the diagram. It is also clear that the amount of evaporation was more harmful when it was experienced during the longer exposures. Lines joining points of equal mortality would be vertical if the duration of exposure had no influence on the mortality rate. In this experiment temperature was probably the least important factor directly influencing mortality; there is, however, a suggestion in Fig. 3 that equivalent amounts of evaporation at 40° C. were more harmful than at 35° C. or 30° C.

Eggs with embryos in an advanced stage of development can hatch at a relative humidity as low as 22 p.c. (20° C.), though optimum conditions are provided by a moist surface. Nymphs hatching in dry soil may be unable to lift the lid of the pod until the surface of the soil is moistened. Eggs of other Acridids can hatch at low relative humidities (Bodenheimer, 1929; Parker, 1930).

SUMMARY.

The water content of turgid eggs of *Austroicetes cruciata* rises throughout development. The increase is slight during diapause but is greater in post-diapause eggs. The movement of water into the eggs can be explained in terms of physical laws. A water-absorbing area or "hydropyle" area is present at the posterior pole of the egg.

The susceptibility of eggs to desiccation, as measured by the rate of loss of water in atmospheres of different humidities, varies according to the stage of development of the embryo, being greatest for newly-laid eggs, and least for diapause eggs. It became progressively greater for eggs collected from the field after mid-May. This relationship has been illustrated by plotting the values for B in an orthogonal polynomial equation against time. The difference between pre-diapause and diapause eggs is associated with the absence of both the yellow and white cuticle in the pre-diapause eggs. The sudden increase in susceptibility to desiccation which occurred in eggs in the field at the end of May, is correlated with the elimination of diapause in the egg and an increase in water content. This change could not be related to differences in the appearance of sections of the egg membranes.

Eggs in the post-diapause state were more easily killed by desiccation than eggs in diapause. However, at all stages of development, prolonged exposure to low humidity was necessary to kill even 50 p.c. of the eggs. In the laboratory some eggs survived with a *water content*: *dry weight* ratio as low as 0·6.

The mortality rate of eggs in diapause was shown to be related to total evaporation and the duration of the exposure to desiccation.

Eggs with embryos in an advanced stage of development can hatch at a relative humidity as low as 22 p.c. (20° C.), though optimum conditions are provided by a moist surface.

Acknowledgments. The authors are indebted to Professor J. Davidson for his help in planning this work, and for valuable criticism and advice in the preparation of the manuscript.

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THE INFLUENCE OF SODIUM ISO-AMYL-ETHYL BARBITURATE UPON THE LIBERATION OF ACETYLCHOLINE FROM THE BOWEL FOLLOWING NERVE STIMULATION

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INTRODUCTION.

Since Loewi (1921) showed that a cardio-inhibitory substance was freed into the heart cavity by vagal stimulation numerous workers have extended the principle of chemical transmission by autonomic nerve stimulation, and Dale (1934) has designated the nerves cholinergic and adrenergic depending on the chemical substance liberated peripherally. Further, Dale and Feldberg (1934) have shown that the chemical transmitter, almost certainly acetylcholine, passes into the venous outflow from the stomach of the dog and that this is increased on vagal stimulation. It has been shown by Wright, Florey and Jennings (1938) that barbiturate drugs cause inhibition of movement of and secretion from the colon following pelvic nerve stimulation, and the evidence points to the probability that acetylcholine is also the transmitter here. Investigation of the secretion of the small intestine (Wright, Jennings, Florey and Lium (1940)) showed that certain anaesthetics abolish or considerably reduce secretory activity produced by nerve stimulation or by the injection of eserine and acetylcholine. It was suggested by Professor Wright that direct tests might be made concerning the influence of barbiturate drugs on the liberation of acetylcholine following nerve stimulation. The present investigation has been designed to determine if sodium iso-amyl-ethyl barbiturate influences the liberation of acetylcholine from the artificially perfused bowel of the guinea-pig following vagal stimulation.

METHOD.

Experiments on Guinea-pigs.

The guinea-pig was stunned by a light blow on the head and a cannula was introduced into the thoracic aorta pointing distally. This was connected to a Dale-Schuster pump operating at 148 cycles per minute and the fluid used was oxygenated Locke Solution (39° C.) to which eserine sulphate was added in a concentration of 1: 140,000. The aorta and inferior vena cava were ligated below the inferior mesenteric branch, the colon and caecum were resected, and a cannula was introduced into the ileal stump. A cannula was introduced into the portal vein from which perfusate was collected by a rubber tube, and the renal pedicles, and the splenic pedicle with the left half of the pancreas were ligated. When a comparison was desired between the effect of stimulation of either vagus, the vagi were isolated in the neck and stimulation for 40 seconds was effected via metal electrodes using an induction coil with the secondary set at 4 cm. using two dry cells. The intestines were kept moist by a cotton wool saline pack and warmed by a 60W electric light globe. In all other experiments the vagi were isolated in the thorax and both were placed between metal electrodes enclosed in a gutta-percha mould round which thin rubber was wrapped and tied to make the capsule water tight and the guinea-pig, after amputation of the limbs, was enclosed in a bath of oxygenated Locke Solution (38–39° C.). Stimulation was applied from a valve stimulator using square waves, frequency 30 cycles per second, and at 15 volts. Perfusate was collected for 14–21 minutes before and for four successive seven-minute periods following stimulation. Stimulation was applied for 40 seconds each minute for 5, 9 or 15 minutes. In some experiments the splanchnics were cut.

Assay of Acetylcholine.

The frog rectus preparation (Chang and Gaddum, 1932) was used for titrating the acetylcholine content of perfusate. In some experiments the assay was made on the rectus abdominis

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prepared similarly from the Hawaiian toad (Bufo marinus). This preparation is sensitive, when treated with eserine in a concentration of 1: 200,000, to acetylcholine in a concentration of 1: 100 million. Further it does not contract to eserine (1: 100,000), histamine (in a concentration up to 1: 100,000) nor to adenosine (1: 100,000).

RESULTS.

1. The Liberation of Acetylcholine following Vagal Stimulation.

When the small bowel and stomach of the guinea-pig are perfused via the aorta with oxygenated eserinized Locke Solution (39° C.) at a rate of 0·5–1 c.c. per min., the vagi having been isolated, the perfusate obtained from the portal vein contains acetylcholine varying in concentration from 1 in 30 to 1 in 8 millions when the whole animal is kept in a bath of oxygenated Locke Solution at 38–39° C. When the animal is not so immersed, the content varies from 1 in 50 to 1: 15 millions acetylcholine. Under the conditions of the experiment, the animal having been starved for 18–24 hours, the variation in acetylcholine content of samples collected did not vary by more than 15 p.c. from sample to sample in four control experiments where collections were made for 14 minute and 7 minute intervals over a period of one hour. There was a similar output of acetylcholine when the vagi were not dissected in control animals.

When the vagus was stimulated there was an increase in the acetylcholine content in the fluid obtained from the portal vein. When the vagi were stimulated separately in the neck it was found that sometimes one vagus and sometimes the other was more effective in liberating acetylcholine, and sometimes they were equal in effect. The maximum increase in acetylcholine concentration in a single sample varied from 80–500 p.c. following stimulation. Where the animal was immersed in a warm bath and the vagi stimulated simultaneously the relative increase was from 100–300 p.c., and the absolute value obtained for the concentration of acetylcholine following stimulation varied from 1: 7·5 to 1: 4·5 millions.

These findings are illustrated in the following experiment. Stomach and small bowel in a guinea-pig weighing 495 gm. were perfused via the aorta with eserinized Locke Solution (1: 140,000 eserine), the splanchnics cut and the animal immersed in a bath of oxygenated Locke Solution at 38° C. 11 c.c. of perfusate were collected during 14 minutes and this when diluted (0·35 c.c. distilled water to 1 c.c. sample) showed activity equivalent to 1: 19·5 millions acetylcholine when tested on the frog rectus. Both vagi were then stimulated for 40 seconds each minute for 15 minutes and the first sample after commencing stimulation (6·5 c.c. in 7 minutes) contained activity equivalent to 1: 12·5 millions acetylcholine. The second, third and fourth samples (each 6 c.c.) collected for successive 7 minute periods contained activity equivalent to 1: 11, 1: 10·5 and 1: 12 millions acetylcholine respectively.

An indication of the increased output can be obtained by comparing the amount of acetylcholine present in the sample obtained for 21 minutes before stimulation, and that in the first three samples after commencing stimulation. The maximal concentration occurred in the second sample, obtained 7–14 minutes after the beginning of stimulation, when this was maintained for 5 or 9 minutes and in the 14–21 minute sample when stimulation was continued for 15 minutes. These results are shown in Table 1. The increase in acetylcholine is seen to vary from 0·4 to 1·3γ, which gives an average increase for each stimulation of 40 seconds of 0·1γ (last column in Table 1). In all these experiments recorded in Table 1 both vagi were placed on the electrodes for stimulation at 30 cycles per second and the animal was immersed in a warm bath. In all cases except that of line three where stimulation was applied in the cervical region, the vagi were isolated in the thorax.

TABLE 1.

Output of acetylcholine from the portal vein of the perfused guinea-pig for 21 min. (A) before and 21 min. (B) after commencing vagal stimulation (38–39° C.).

Weight pig.	Time of stimulation in minutes.	A γ	B γ	Output acetylcholine γ per 40" stimulation.
690 gm.*	—	1·7	1·4	—
660	—	1·8	1·3	—
592	5	1·9	2·4	0·1
500	9	1·3	1·7	0·05
520	9	0·9	1·7	0·09
480 S	9	0·7	1·7	0·11
495 S	15	1·05	2·3	0·09
660 S B	15	1·1	0·9	−0·013
650 S B	15	1·0	1·2	+0·013
665	15	1·2	1·1	−0·007
635 B	15	0·34	0·39	+0·003

* = Vagi not dissected. S = Splanchnics cut. B = Barbiturate perfusion.

In Fig. 1 are shown the responses of the frog rectus to samples from the experiment of line 3 (left hand panel) and line 1 (right hand panel). The samples obtained 0–7, 7–14 and 14–21 minutes after stimulation (2, 3, 4) are seen to contain increased amounts of acetylcholine compared to that obtained before stimulation (1). The right hand panel shows the responses to samples obtained in the absence of vagal stimulation at 1 of the sample collected for the first 14 minutes, and at 3 and 5 samples collected 7–14 and 21–28 minutes later. The concentrations are shown in graphic form in Fig. 2 where the continuous line shows the increase in concentration following vagal stimulation, and the interrupted line of slight fall in the absence of stimulation.

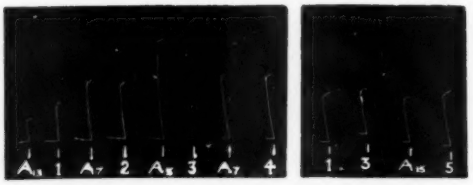

Fig. 1. Responses of the frog rectus to samples obtained from the perfused guinea-pig bowel. Left-hand panel: 1, the responses to the sample collected 14 minutes before, and 2, 3 and 4 successive samples obtained 0–7, 7–14 and 14–21 minutes after the onset of stimulation of both vagi for 40 seconds each minute for five minutes. At A_{13}, A_7, A_5, acetylcholine 1: 13, 1: 7 and 1: 5 millions were tested. Right-hand panel: the responses to samples collected from another guinea-pig in the absence of vagal stimulation in the first 14 minutes at 1, 21–28 minutes at 3 and 35–42 minutes at 5. The samples collected 14–21 minutes and 28–35 minutes contained slightly less acetylcholine than sample 1. At A_{15} the response to acetylcholine 1: 15 millions.

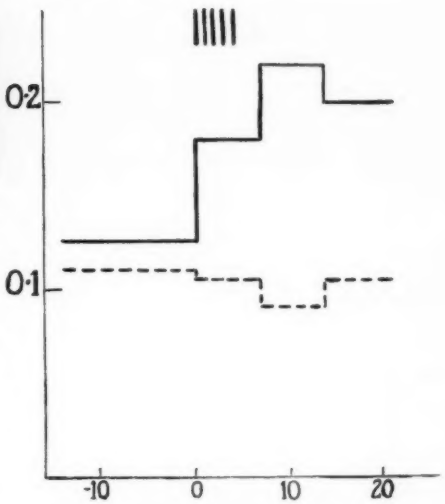

Fig. 2. Concentration of acetylcholine in portal perfusate from two guinea-pigs immersed in a warm bath; continuous line, vagal stimulation at the vertical lines; interrupted line, no stimulation performed, ordinates: acetylcholine in γ per c.c., abscissae: time in minutes. Details in text.

2. Inhibition of the Liberation of Acetylcholine by Vagal Stimulation in the presence of Barbiturate.

When the stomach and intestine are perfused with eserinized Locke Solution to which sodium-iso-amyl-ethyl barbiturate is added (0·06 mg. per c.c. solution) there is still a continuous output of acetylcholine into the portal vessels varying from less than 1: 60 millions to 1: 50 millions in the pigs which were not immersed in a warm bath, and from 1: 34 to 1: 9 millions where they were immersed in a warm bath. Following vagal stimulation there is no significant output of acetylcholine. Barbiturate in the concentration in the samples tested on the frog rectus (0·044 mg. per c.c.) (they were diluted 35 p.c. to reduce osmotic pressure) does not cause contraction of the muscle and was without effect on the size of the response to doses of acetylcholine of the order estimated. In the case of the toad sometimes a diminution of 5 p.c. was observed in the size of the response. Twice the concentration of barbiturate (0·088 mg. per c.c.) produced depression of the response with both preparations.

The findings in two experiments when vagal stimulation was applied first to the left vagus and then to the right are shown in Fig. 3. The continuous line shows the output following induction-coil stimulation of the vagi each for 40 seconds in a pig weighing 315 gm. perfused with eserinized oxygenated Locke Solution at 39° C. The interrupted line shows the findings where a pig weighing 402 gm. was perfused with barbiturate in eserinized oxygenated Locke Solution, and the same stimulation applied. It is seen that when barbiturate was used there was no significant output of acetylcholine. This was obtained regularly, but since induction-coil stimulation is not constant in its output a better comparison was effected by stimulating with square waves at 30 cycles per sec. at a fixed voltage from a valve stimulator (see methods) though here

both vagi were placed in the electrode so that a comparison of the right and left vagi was not obtained. The results of these experiments are shown in Table 1. The depression of output of acetylcholine following barbiturate perfusion (B, column 4) when the vagi were stimulated is well shown. In the experiment of the second last line the sample obtained 7–14 minutes after cessation of stimulation showed an increase in acetylcholine content from 1: 8 millions to 1: 4·5 millions.

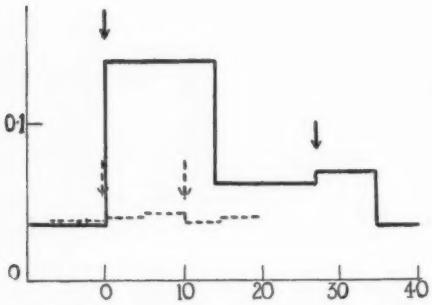

Fig. 3. Concentration of acetylcholine in portal perfusate from two guinea-pigs. At the arrows stimulation of the left and right vagi respectively for 40 seconds. Continuous line, barbiturate-free perfusion fluid. Interrupted line, barbiturate in perfusing fluid. Ordinates and abscissae as in Fig. 2. Details in text.

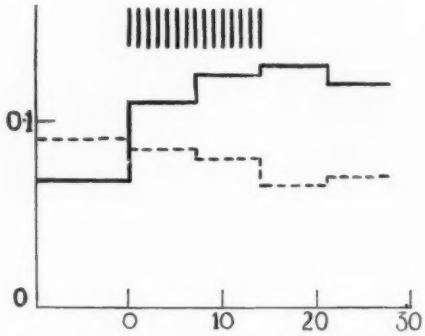

Fig. 4. Concentration of acetylcholine in perfusate from portal vein from two guinea-pigs; continuous line, barbiturate-free perfusing fluid; interrupted line, barbiturate in perfusing fluid. Vagal stimulation at the vertical lines in each. Ordinates and abscissae as in Fig. 2. Details in text.

Although it was unlikely that the thoracic sympathetic cord was viable in these experiments, some experiments were performed where the splanchnic nerves were divided. (8 in Table 1). The results were similar. The findings in two experiments when the guinea-pig was placed in the bath of Locke Solution are shown in Fig. 4. Stimulation was applied to both vagi for 40 seconds each minute for 15 minutes at 30 cycles per second (15 volts), the splanchnic nerves being divided. In the one (continuous line) where a guinea-pig weighing 495 gm. was perfused with oxygenated eserinized Locke Solution and the splanchnics divided, the gut was very quiescent at the beginning of the experiment and it was not till the sixth stimulation that there was a good contraction of a loop of bowel which occurred after a latency of 12 seconds. Contractions occurred following further stimulation after latencies of from 15–20 seconds. The output of acetylcholine is shown by the continuous line in Fig. 4, and shows an increase following stimulation. In the other experiment (interrupted line) a guinea-pig weighing 660 gm. was perfused with barbiturate in oxygenated eserinized Locke Solution. The gut was contracting fairly actively both before and after stimulation. A progressive slight fall in the output of acetylcholine following stimulation is shown by the interrupted line in Fig. 4. The responses of the frog rectus to samples of fluid from these two experiments when diluted with distilled water are shown in Fig. 5. The upper panel (barbiturate perfusion) shows a slight decline in the acetylcholine concentration after stimulation (2, 3, 4 and 5 samples obtained 0–7, 7–14, 14–21 and 21–28 minutes respectively after stimulation); the lower panel shows a considerable increase after stimulation when no barbiturate was used.

DISCUSSION.

The continuous output of acetylcholine into the portal vein from the stomach and small bowel perfused with eserine may be related to the bowel movements which occur continually and may not in this instance be due to activity in digestive glands since the animals were kept without food for 18–24 hours before experiments. Dale and Feldberg (1934) have shown previously that there is a continuous small output of acetylcholine from the stomach of the dog and that the output increases to $2\frac{1}{2}$ to 4 times following vagal stimulation. We obtain similar results with the guinea-pig. In our experiments, however, we were unable completely to excise

the pancreas owing to its intimate relation with the duodenum ,and this small amount of tissue may have contributed to the output.

The depression of movement and secretion of the colon in the cat in the presence of barbiturate which normally occurs on stimulating the pelvic nerve (Wright, Florey and Jennings, 1938) suggests that barbiturate may either inhibit the ouput of acetylcholine or abolish the response of the bowel to acetylcholine. We have shown that barbiturate inhibits the liberation of acetylcholine following vagal stimulation. With the same dose of barbiturate we also note a slight depression in the response of the toad rectus to acetylcholine which is increased with higher concentrations of barbiturate.

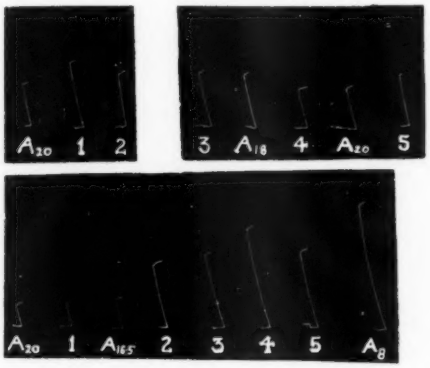

Fig. 5. Responses of the frog rectus to samples obtained from the perfused bowel of the guinea-pig, splanchnics divided. Upper panel: barbiturate in perfusing fluid, at 1, 2, 3, 4 and 5 the responses to samples 14 minutes before, and 0–7, 7–14, 14–21 and 21–28 minutes respectively after the beginning of stimulation of the vagi for 40 seconds each minute for 15 minutes. At A_{15}, A_{18} and A_{20} responses to acetylcholine 1: 15, 1: 18 and 1: 20 millions. Lower panel: oxygenated eserinized Locke Solution alone as perfusing fluid; at 1, 2, 3, 4 and 5 responses to samples obtained similarly to above. At A_{20}, $A_{16·5}$ and A_8 the responses to acetylcholine 1: 20, 1: 16·5 and 1: 8 millions. Further details in text.

Bayliss and Starling (1899) proved conclusively that vagal stimulation caused contraction of the small bowel when the splanchnic nerves were cut and that this increased with repeated stimulation. In view of the fact that we found a fairly constant output of acetylcholine for each 40 seconds stimulation whether this was maintained from 5, 9 or 15 minutes and that we similarly obtained an increased response with repeated stimulation, we suggest that if acetylcholine is the transmitter there is some change in the sensitivity of the bowel to acetylcholine, rather than that there is an increasing output of acetylcholine following successive stimulations.

From an analysis of two curves, the unit time and the single stimulus-salivary secretion curve, Beznák and Farkas (1937) were able to show that acetylcholine causes an increased sensitivity of the gland cell to the drug in investigations on salivary secretion caused by direct nerve stimulation. Clark and Raventós (1938) observed similar phenomena with the auricle and found that they could explain their results in the same way. It is noteworthy that Rosenblueth and Cannon (1940) have shown that there is an increased response of skeletal muscle to acetylcholine after tetanization and suggest that it may be due to mobilization of KCl during tetanus.

There was a marked variation in the degree of motility of the gut at the beginning of the experiment from animal to animal. The initial inhibition of movement following vagal stimulation as described by Bayliss and Starling (1899) was readily detected. We have noted the change in movement following stimulation and have found that usually (five experiments) one more loop of bowel was contracting following stimulation than before. Once no increase was seen, and twice, three and four more coils were observed to contract following stimulation. In the presence of barbiturate twice a slight increase in movement was noted following stimulation, but in four other experiments no increase at all was observed. In one experiment when the gut was contracting well before stimulation initial inhibition following vagal stimulation in the presence of barbiturate was well shown. We suggest it is possible that the use of barbiturates in the dosage of 0·06 gm. per litre

of circulating fluid is inimical to the liberation of acetylcholine and may play a part in the depression of bowel movements following operative interference where this drug has been used for preliminary medication.

SUMMARY.

When the stomach and small bowel of a guinea-pig are perfused with eserinized Locke Solution a substance is detected in the effluent from the portal vein which causes contraction of the isolated rectus muscle of the frog and of the toad.

This substance is increased in amount following vagal stimulation.

In the presence of sodium iso-amyl-ethyl barbiturate (0·06 mg. per c.c.) this output following nerve stimulation is inhibited.

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INSULIN INACTIVATION BY SULPHYDRYL COMPOUNDS

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In a previous paper (Corkill and Ennor, 1940), the view was expressed, contrary to that of Jacobs (1938), that the inhibitory effect of sulphydryl compounds on insulin hypoglycaemia is not due to inactivation of insulin. Our evidence for this assumption was as follows: (1) The subcutaneous injection of cysteine into the intact animal leads to hyperglycaemia, which would mask the hypoglycaemic action of injected insulin. (2) In the spinal eviscerated cat, cysteine does not inhibit the hypoglycaemic action of insulin. (3) No correlation could be found between the reduced glutathione content of rabbit's liver and insulin sensitivity.

The experiments described in this paper suggest that cysteine hyperglycaemia is due to a reactionary output of adrenaline from the suprarenal glands.

Experiments with Cysteine and Ergotoxine.

Ergotoxine abolishes the motor effects of sympathetic activity and, if our theory is correct, cysteine should not produce hyperglycaemia in animals previously given ergotoxine. For such experiments adult English hutch rabbits, deprived of food for 24 hours, were used. 5 mg. of ergotoxine ethanesulphonate (Burroughs Wellcome) were injected intravenously. Thirty minutes later blood for glucose estimation was taken and immediately afterwards cysteine (0·6 gm. per kg.) was injected subcutaneously. Blood samples were taken 60 and 90 minutes later. True blood glucose was determined as described in the previous paper. The results are shown in Table 1.

TABLE 1.

Effect of ergotoxine on cysteine hyperglycaemia.

Blood glucose, mg. p.c.

Time in minutes.	Controls, no ergotoxine.		Ergotoxine and cysteine.			
0	117	104	102	117	131	117
cysteine →						
60	165	145	108	104	136	—
90	157	187	102	112	131	110

Unlike the controls, the animals treated with ergotoxine did not exhibit hyperglycaemia when injected with cysteine.

The Influence of Cysteine on Insulin Hypoglycaemia in Adrenalectomized Rabbits.

Adrenalectomized rabbits are, in a sense, comparable to those treated with ergotoxine, since hyperglycaemia could not be due to a secondary output of adrenaline.

Bilaterally adrenalectomized rabbits were prepared as described in our previous paper. In a preliminary test the animals were fasted for 16 hours and the blood sugar response to a small dose of insulin determined. Following an interval of 5–8 days, cysteine was administered and the blood sugar response to the same amount of insulin again determined. The amount of cysteine given was 0·6 gm. per kg. body weight; in animals 1 and 2, ⅔rds of the total amount were injected 10 minutes before the intravenous injection of insulin, and the remainder 20 minutes after insulin. In animals 3–6 cysteine was given 30 minutes before and 10 minutes after insulin. These results are shown in Table 2.

These results indicate clearly that cysteine has not diminished the hypoglycaemic action of insulin. This is particularly noticeable with animals 3–6 inclusive, in which 1/40 unit of insulin produced a slight but definite hypoglycaemia despite the increased ratio of cysteine to insulin. The amount of cysteine injected into this group of animals will produce marked hyperglycaemia in intact animals. Its failure to mask the mild hypoglycaemia due to 1/40 unit of insulin affords confirmatory evidence of the rôle played by the suprarenal glands in cysteine hyperglycaemia.

[1] Working under a full time grant from the National Health and Medical Research Council.

TABLE 2.

Adrenalectomized rabbits.

Blood glucose, mg. p.c.

Rabbit No.	Insulin dose, units.		Time in minutes after injection of insulin. 0	10	20	30	40	50	60	90	120
1	1/8	"a"	132	—	—	59	—	—	84	88	99
		"b"	134	—	—	61	—	—	51	75	90
2	1/4	"a"	115	—	—	52	30 ← convulsions				
		"b"	104	—	—	32 ← convulsions					
3	1/40	"a"	111	104	87	94	108	114			
		"b"	118	86	81	84	91	91			
4	1/40	"a"	97	77	72	59	64	70			
		"b"	102	78	72	60	60	66			
5	1/40	"a"	106	93	83	81	101	110			
		"b"	104	95	82	83	101	97			
6	1/40	"a"	110	99	83	97	115	113			
		"b"	108	95	93	83	93	95			

"a" = insulin alone; "b" = cysteine and insulin.

SUMMARY.

Cysteine, although producing hyperglycaemia in the intact animal, fails to do so in rabbits injected with ergotoxine.

In adrenalectomized rabbits injection of cysteine does not diminish the hypoglycaemic action of insulin.

It is suggested that the apparent inactivation of insulin by cysteine in the intact animal is due to the fact that cysteine hyperglycaemia masks the hypoglycaemic action of insulin.

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THE INFLUENCE OF TEMPERATURES ABOVE THE DEVELOPMENTAL ZERO ON THE DEVELOPMENT OF THE EGGS OF *AUSTROICETES CRUCIATA* SAUSS. (ORTHOPTERA)

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INTRODUCTION.

The small plague grasshopper *Austroicetes cruciata* Sauss. is distributed in a belt across Australia (Key, 1938). In South Australia the eggs are laid into the soil in November and hatch in the following September (Andrewartha, 1939). They are characterized by a diapause which inhibits development until they have been exposed to the low temperatures of autumn and winter: although thousands of eggs, in the early stages of diapause, have been incubated at constant temperatures from 25° C. to 30° C., none has hatched. In this respect the eggs resemble those of *Circotettix verruculatus* (Carothers, 1923) and differ from those of *Melanoplus differentialis* (Bodine, 1925).

The experiments recorded in this paper were done to find out at what time of the year eggs of *A. cruciata* developing in the field become capable of responding normally to temperatures above the "developmental zero". In the eggs of *Melanoplus differentialis* the diapause is eliminated before the onset of the coldest winter months (Nelson, 1930).

The influence of temperatures above the "developmental zero" on the rate of development of eggs in the late stages of diapause, as well as for eggs in which diapause has been eliminated, has been studied. For the latter a curve was obtained relating temperature to the rate of development. Various workers have proposed formulae relating the rate of development of poikilothermic animals to temperature; the relationship, however, is not simple. Although some of these formulae are adequate for particular data, none can be accepted as a satisfactory statement of a general law.

A preliminary experiment was done to find out the influence of moisture on the rate of development. Most workers have found that the rate of development of insect eggs is retarded with dryness, but the effect is small (Mellanby, 1935), except under very dry conditions and in specialized cases such as locust eggs (Husain, Taskhir and Mathur, 1940).

MATERIALS AND METHODS.

The eggs used in these experiments were collected at Wilmington and Orroroo at monthly intervals from mid-February to mid-April, 1940, and thence at fortnightly intervals until July. They were separated from the pods and five lots of thirty eggs were used for each treatment. Each lot was placed in a celluloid container which had a plaster of Paris bottom and cellophane cap. The plaster of Paris was kept moist by resting the containers in petri dishes containing a little water; this was found to provide favourable moisture for the eggs.

Nine incubators were used; temperature was controlled within $\pm 0{\cdot}1°$ C. in six incubators and $\pm 0{\cdot}2°$ C. in the other three. Wet and dry bulb readings were taken in each incubator; the temperature at the surface of the plaster of Paris was also taken, this was considered to approximate to that experienced by the eggs. The difference between the plaster of Paris and dry bulb temperatures varied from $0{\cdot}2°$ C., when the dry bulb temperature in the incubator was $16{\cdot}3°$, to $2°$ C. when the dry bulb temperature was $34{\cdot}9°$ C. The mean temperature for each experiment was calculated from the maximum and minimum temperatures of the thermostat cycle.

The eggs were incubated at 30° C. as soon after collecting as possible. After 30 days' incubation ten eggs were fixed in hot Bouin's fixative and stained *in toto* in borax carmine; the remaining eggs were then transferred to the open compartment of the glasshouse, the plaster

of Paris on which the eggs were resting being kept continually moist. Eggs collected at the end of April and all subsequent dates were incubated at seven constant and four alternating temperatures; these eggs were discarded after one month if the embryo failed to complete katatrepsis[1] in this time. The fortnightly collecting of eggs was continued until diapause had been eliminated from eggs in the field. Hatchings were counted at regular intervals thrice daily for the five higher temperatures and twice daily for the lower temperatures. An analysis of the results showed that the loss of information caused by these group intervals was less than 1 p.c. for the two temperatures 18·9° C. and 16·1° C. Group intervals twice the size of those used would have been sufficient at 24·3° C. and 22·5° C., whereas four-hourly readings would have been necessary at 30·5° C. and 27·0° C. to give the same accuracy. No figure was obtained for the loss of information with the group interval used at 32·9° C., because of the marked skewness of the distribution of hatchings at that temperature.

RESULTS.

The Influence of Temperature on the Development of Eggs in Late Diapause.

Some eggs from those collected in mid-May were the first to complete development at temperatures between 18·9 and 27·1° C.; at 30° C. and over, none of the embryos completed katatrepsis; more embryos completed katatrepsis at the lower temperatures. Most eggs completing katatrepsis hatched. The wide range about the mean of the incubation period at the lower temperatures indicates considerable variability of the eggs in response to these temperatures at this stage of development.

Some eggs (66 p.c.) collected two weeks later (May 29th) were the first to hatch at 30° C. At 32° C. only 19 p.c. hatched. More eggs hatched at the lower temperatures, and there was still a wide scatter about the mean incubation period. These eggs responded to temperature in a normal way after they had been exposed to 8° C. and 13·5° C. on alternate days for 23 days (Table 5). The rates of development of eggs in these two stages of late diapause are shown in Fig. 1 and Table 1.

TABLE 1.

Showing the influence of temperature on the development of eggs collected on May 16th and May 29th.

Temperature ° C.	P.c. eggs completed katatrepsis.	P.c. eggs hatched.	Days to hatch.	Range of hatching (days).
		May 16th.		
16·1	25	0	—	—
18·9	20	16	47	37–60
22·5	5	5	25	24–27
24·4	9	9	19	18–21
27·1	7	3	19	18–21
30·3	2	0	—	—
32·0	0	0	—	—
		May 29th.		
16·1	—	92·5	55·8	47–80
18·9	—	94·5	31·4	27–42
22·3	—	68·0	17·9	17–19
24·4	—	76·0	14·0	11–18
27·0	—	74·0	11·7	9–15
30·3	—	66·5	7·9	6–12
32·0	—	19·0	—	6–11

The Elimination of Diapause in the Field.

Diapause was considered to have been eliminated when the eggs were able to hatch on being incubated at 30° C. This temperature was chosen because it does not have any harmful effect on post-diapause eggs, and it appears to have no influence on the elimination of diapause. Table 1 shows that some eggs in late diapause which were unable to hatch at 30° C. did so at lower temperatures.

The period during which diapause was being eliminated in the field is shown in Tables 2 and 3. The stages of development of the embryos referred to in this section correspond to those described by Steele (1941).

[1] The term "katatrepsis" was coined by Wheeler (1893). It is now used to describe the second stage of blastokinesis in which the embryo migrates head first towards the anterior pole of the egg, taking up a position with its ventral surface against the ventral surface of the egg.

The difference between the percentage of eggs which had completed katatrepsis by May 29th at Wilmington and Orroroo (8 p.c. and 17 p.c.) may not be real; 4,250 eggs were examined from Wilmington but only 280 eggs from Orroroo.

The eggs which were collected from Orroroo on May 16th developed to a greater extent at 30° C. than eggs collected at Wilmington on the same day (Table 3). By the end of May the percentage hatch and mean time to hatch at 30° C. were not significantly different from those for eggs collected at Wilmington. Diapause appears to have been eliminated more rapidly at Orroroo; this is probably associated with the lower temperature due to greater altitude, 1,380′ compared to 1,060′ at Wilmington. The more gradual completion of katatrepsis in the field and the slower increase in percentage hatch are explained on the same grounds. The resultant effect of more rapid elimination of diapause and slower post-diapause development brings the eggs in the two localities to a comparable stage by the end of May and to "normality" by mid-June.

The eggs which had overwintered in the glasshouse hatched despite the earlier incubation for 30 days at 30° C.

TABLE 2.

Showing the stage reached by the embryo in eggs collected in the field on various dates, and the ability of the embryo to develop further at 30° C.

Date collected from field.	Stage of development when collected.	Stage of development after 30 days at 30° C.	Remarks.
February 16th	I–IV	VIII	Embryos increased 2 to 3 times in length.
March 11th	III–VI	IX–X	Slightly greater development than that of the previous collection.
April 16th	VIII–IX	X	Relatively little development.
April 30th	IX	X	Relatively little development.
May 16th	X	XIV	Appendages developed to stage XIV without, however, either yolk engulfment or katatrepsis having been completed. Katatrepsis had started in 2 p.c.
May 29th	X–XIII	XIV–XVI	17 p.c. of the embryos were in the process of katatrepsis (stages XI–XIII). 382 eggs were incubated. 82 eggs in which katatrepsis had been completed in the field hatched in 6·6 days (s.e. = 0·067). 89 p.c. of the remaining 300 eggs with eyespots at the posterior pole completed katatrepsis; 66 p.c. hatched in 7·9 days (s.e. = 0·106) and 23 p.c. were unable to hatch although they had completed katatrepsis and made considerable development.
June 16th	XIV	XVI	96 p.c. hatched in 5 days.
July 18th	XV	XVI	89 p.c. hatched in 4·9 days.

TABLE 3.

Showing the ability of eggs collected at Wilmington and Orroroo at various dates, to complete katatrepsis and hatch at 30° C.

	Wilmington.			Orroroo.		
Date collected from field.	P.c. eggs completed katatrepsis at 30° C.	P.c. eggs hatched at 30° C.	Days to hatch at 30° C.	P.c. eggs completed katatrepsis at 30° C.	P.c. eggs hatched at 30° C.	Days to hatch at 30° C.
Feb. 16th	0	0	—	—	—	—
March 11th	0	0	—	0	0	—
April 16th	0	0	—	0	0	—
April 30th	0	0	—	0	0	—
May 16th	0	0	—	3	30	10·2 ± 0·266
May 29th	17	67	7·9 ± 0·106	8	74	7·7 ± 0·098
June 16th	93	96	*5·0 ± 0·036	95	97	5·5 ± 0·109
July 18th	97	89	*4·9 ± 0·096	100	87	4·5 ± 0·126

± = Standard error. * = Significantly different from corresponding figure for Orroroo.

The Influence of Temperature on the Rate of Development of Post-Diapause Eggs.

(1) *The effect of moisture.* In a preliminary experiment a comparison was made of the rate of development of eggs incubated on moist plaster of Paris and in air having relative humidities of 100 p.c., 90 p.c. and 70 p.c. at 18·9° C.

TABLE 4.

Showing the influence of moisture on the rate of development of eggs when incubated at 18·9° C.

Moisture conditions.	P.c. eggs hatched.	Days to hatch.	Standard error (days).
Moist plaster of Paris	95	22·8	0·3007
100 p.c. R.H.	88	21·4	0·1928
90 p.c. R.H.	78	21·5	0·1736
70 p.c. R.H.	82	22·6	0·2610

The eggs used in this experiment were collected at Wilmington on April 30th, they were placed on moist plaster of Paris at 13·5° and 8° C. on alternate days for 56 days to eliminate diapause; these eggs had absorbed water to the maximum of their capacity. They were then incubated at 18·9° C. under the four conditions of moisture shown in Table 4. As soon as hatching began the eggs incubated in air were placed on moist plaster of Paris; but at 100 p.c. R.H. 40 p.c. of the eggs hatched before they were transferred to the moist plaster of Paris; at 90 p.c. R.H. and 70 p.c. R.H. 32 p.c. and 12 p.c. hatched before being moistened.

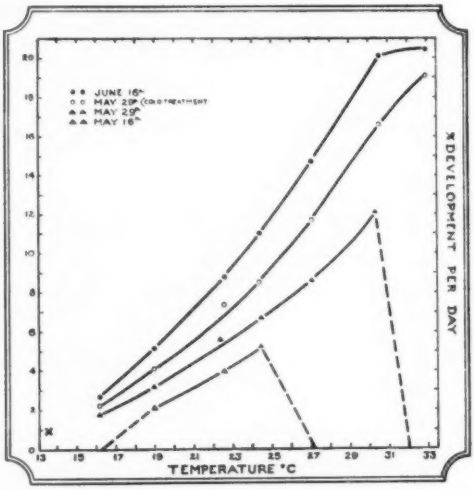

Fig. 1. Showing the rate of development in relation to temperature for eggs of *A. cruciata* collected from the field at various dates. × indicates the rate of development at 13·5° C. (This point was derived from an experiment using alternating temperatures (p. 8).) The points have been joined by freehand curves.

Eggs at 100 p.c. R.H. and 90 p.c. R.H. hatched 1·3 to 1·4 days earlier than those resting on plaster of Paris throughout the experiment. This difference is significant. The time for hatching at 70 p.c. R.H. was not significantly different from that for eggs incubated on moist plaster of Paris, though significantly greater than at 90 p.c. and 100 p.c. R.H. The figures are difficult to interpret since the exact temperature experienced by the eggs is not known. The slower rate of development of the eggs on the plaster of Paris may have been due to a lower temperature on the moist surface produced by evaporation. The difference between the wet and dry bulb readings is this incubator was 0·7° C. A temperature difference of 0·7° C. corresponds to a difference of 1·6 days in the duration of the incubation period. The difference in time of hatching between eggs incubated on the moist plaster of Paris and those incubated in an atmosphere of 100 p.c. R.H. was 1·4 days, hence the difference in rate of development could be accounted for if we consider the wet bulb readings to represent the temperature experienced by the eggs on the moist plaster of Paris.

The results show that the influence of moisture on the rate of development is not great. Parker (1930) and Bodine (1925) obtained similar results with the eggs of American grasshoppers.

(2) *The rate of development at constant temperatures.* Two curves relating the rate of development and temperature were obtained for post-diapause eggs, one curve for eggs collected at Wilmington on May 29th and the other for eggs collected on June 16th.

With the eggs collected on May 29th only those eggs were used in which the embryo had not yet commenced katatrepsis. These eggs were subjected to a cold treatment at 13·5° C. and 8° C. on alternate days for 23 days. Within this time the eyespots had moved to the anterior poles of the eggs in a uniform manner. The eggs collected on June 16th were exposed to outdoor temperatures for three days during transit to the laboratory; a further day was spent at 13·5° C. while the eggs were being dissected from the pods: all the eggs were in a similar stage of development at the beginning of the experiment.

The results of the experiments with both lots of eggs are shown in Table 5 and Fig. 1.

TABLE 5.

Showing the rate of development of post-diapause eggs collected in the field on May 29th and June 16th.

Temp. ° C.		Eggs collected May 29th (cold treatment).				Eggs collected June 16th.			
Mean.	Range. ± ° C.	P.c. hatched.	Days to hatch.	P.c. dev. per day.	Standard error.	P.c. hatched.	Days to hatch.	P.c. dev. per day.	Standard error.
16·1	0·1	89·5	45·0	2·24	0·002	99·0	38·6	2·69	0·026
18·9	0·2	91·5	24·4	4·12	0·030	91·0	22·7	5·18	0·059
22·5	0·1	98·0	13·7	7·35	0·065	90·5	11·6	8·78	0·104
24·3	0·2	96·0	11·3	8·53	0·041	98·0	9·2	11·06	0·112
27·0	0·2	91·0	8·6	11·68	0·087	98·5	6·9	14·72	0·132
30·5	0·2	87·5	6·1	16·55	0·144	96·0	5·0	20·11	0·146
32·9	0·7	72·0	5·3	19·11	0·173	90·5	5·0	20·42	0·205

(3) *The rate of development at alternating temperatures.* Experiments with alternating temperatures were planned to find out, first, whether the rate of development of eggs at any given temperature within the range 16·1° C.–32·5° C. was the same when eggs were incubated constantly at that temperature as when incubated on alternate days at that temperature and another temperature within the above range, and secondly, to find out the rate of development of eggs at 13·5° C. and 8° C.

(i) Eggs collected on May 29th were placed at 13·5° C. and 8° C. on alternate days for 23 days so as to eliminate diapause. These eggs were then incubated on alternate days at two different temperatures within the range 16·1° C.–32·5° C. The following combinations of temperature were used: 18·9° and 30·5° C., 18·9° and 32·9° C., 22·5° and 27·0° C., 22·5° and 30·5° C.

The theoretical rate of development of eggs in terms of the rates at constant temperatures was calculated by applying the mean rate of development per day at constant temperatures to the number of days spent at these temperatures. If the variance of the calculated mean is similar to the variance of the observed mean, then the use of mean rates in the calculation of the theoretical mean is justified. The effect of the faster rate of the first eggs to hatch is then cancelled out by the slower rate of the last eggs to hatch. In this experiment these effects do cancel out (Table 6).

The variance of the calculated mean is: $\frac{1}{N} \cdot \Sigma \frac{d_1^2 S_1 + d_2^2 S_2}{(d_1 + d_2)^2}$, where d_1 = days at temperature 1, d_2 = days at temperature 2, N = number of eggs hatched, S_1 = variance of mean rate for temperature 1 and S_2 = variance of mean rate for temperature 2.

TABLE 6.

Showing the influence of alternating temperatures as compared with constant temperatures on the rate of development of eggs.

High temp. ° C.	Low temp. ° C.	P.c. dev. per day		Variance	
		Observed.	Calculated.	Observed.	Calculated.
30·5	18·9	9·98	9·99	0·0072	0·0049
32·9	18·9	*11·82	*11·14	0·0074	0·0067
27·0	22·5	9·33	9·39	0·0030	0·0025
30·5	22·5	11·83	11·66	0·0075	0·0054

* Significantly different.

The results show that there was no acceleration of development when eggs were incubated alternately at two temperatures within the range of 18·9° C. to 30·5° C. The actual and calculated means are practically identical. The retarding effect obtained at 32·9° C. is evidently less when the eggs were incubated on alternate days at a lower temperature.

(ii) (a) *Development at 13·5° C.* Eggs collected at Wilmington on June 16th were placed at 8° C. and 13·5° C. on alternate days for 36 days. It was considered that after this prolonged exposure to low temperatures there would not be any accelerating effect due to the elimination of any residual diapause during the experiment. One lot of these eggs was then incubated at 13·5° C. for 86 days and subsequently at 18·9° C. After allowing for development at 18·9° C. it was calculated that 0·69 p.c. development per day occurred at 13·5° C. A second lot of eggs was incubated at 13·5° C. and every fourth day at 18·9° C. The first egg hatched after 24 days had been spent at 13·5° C. It was calculated that with the shorter exposure at 13·5° C. 0·98 p.c. development per day occurred at that temperature. A control lot was incubated at 18·9° C.

One egg of 150 eggs collected on May 29th hatched after 138 days at 13·5° C. The remaining eggs were fully developed but were unable to hatch until removed to a higher temperature.

(b) *Development at 8° C.* Eggs collected on July 18th were incubated at 8° C. and every fourth day at 18·9° C. The first egg hatched after 24 days had been spent at 8° C. The difference of 0·07 p.c. between the observed and calculated rates, which represents the rate of development at 8° C., is within the experimental error. However, not all activity ceased at 8° C.: Some eggs in which the eyespots had reached a central position in the egg at 13·5° C. were placed at 8° C.; after five weeks the eyespots had moved one quarter the length of the egg.

DISCUSSION.

The results in this paper have been examined in relation to the work of other authors on the influence of temperature on insect eggs which have a diapause, and in relation to the current theories relating temperature to physiological processes in poikilothermic animals, such as the rate of development, the rate of oviposition and the respiration rate.

The Influence of Temperature on the Development of Eggs in Late Diapause.

With eggs in the late diapause stage (e.g. those collected on May 16 and May 29) exposure to temperatures between 16° C. and 27° C. had some effect in eliminating diapause. The wide range about the mean of the incubation period indicated that the embryo was not yet developing normally at these temperatures (Table 1 and Fig. 1).

Little information is available concerning the eggs of grasshoppers or other insects which fail to hatch when incubated from the time they are laid at constant temperatures above the "developmental zero". More detailed studies have been made on insect eggs having a diapause which is gradually eliminated at temperatures above the developmental zero, e.g. *Melanoplus mexicanus* (Parker, 1930) and *Lymantria monacha* L. (Zwölfer, 1934).

Variability in the time taken for eggs to hatch, similar to that in the late diapause eggs of *A. cruciata*, was demonstrated for the eggs of *M. mexicanus* and *M. differentialis* incubated at constant temperatures from the time they were laid (Parker, 1936; Burdick, 1937). Variability was also found in the partly overwintered eggs of *Lymantria monacha* L. (Goldschmidt, 1932). For all these insects the least variation occurred under conditions producing the fastest rate of development.

The Elimination of Diapause in the Field.

The elimination of diapause in the field proceeded in conjunction with slow development. Although only slight development took place in the field during February and March, eggs collected during these and subsequent months made further development in the laboratory at 30° C. From March until the end of April a progressive development of the embryo took place in eggs in the field as well as at 30° C. in the laboratory. Diapause was thus gradually being eliminated from February onwards. (No eggs were collected before February).

In the embryos which were examined, maximum morphological development was reached about a fortnight before katatrepsis could occur. The final physiological changes which make katatrepsis and subsequent development possible occur during this period of late diapause.

Eggs collected in mid-June were the first to hatch with a normal low mortality rate at all the temperatures used and with a normal low standard error of the mean rate of development. Over 90 p.c. of the eggs had completed katatrepsis in the

field by this date. Under artificial conditions the completion of diapause is not necessarily coincident with katatrepsis, for many eggs in the stage of late diapause (May 29th) completed katatrepsis at 30° C. in the laboratory but failed to hatch. Eighty eggs which had completed katatrepsis in the field by this date hatched normally.

The gradual sequence of response to temperature exhibited by late diapause eggs (Tables 1–3) tends to obscure the fact that "normality" was reached by the eggs in the field with remarkable uniformity and final suddenness. The percentage of eggs which hatched when incubated at 30° C. changed from 0 to 60 within a fortnight; during this period little, if any, morphological development of the embryo took place.

Diapause in the eggs of *Melanoplus differentialis* is eliminated before the onset of winter (Nelson, 1930). It occurs at a much earlier stage of embryonic development than is the case in the eggs of *A. cruciata*. The period of concurrent elimination of diapause and development of the embryo is thus much shorter in *M. differentialis*. The warm temperate climatic zone in which the eggs of *A. cruciata* develop provides sufficient warmth during the late autumn for the embryos to complete katatrepsis by the middle of June. This does not occur until the spring in the eggs of *M. differentialis* (Nelson, 1930) and *Conocephalus (Xiphidium) ensiferus* (Wheeler, 1893). Eggs of *A. cruciata* in diapause collected in the field before May 16th and incubated for a month at 30° C. were unable to develop to the advanced morphological stage which Nelson (1930) found was attained by the eggs of *M. differentialis* at 25° C. The slowing down of growth in the embryo of *A. cruciata* in the field in the fortnight or so prior to katatrepsis may be a general phenomenon in Acridid eggs. Payne (1934) observed that the growth of all parts of the grasshopper embryo slows down before katatrepsis, in eggs which have a diapause and in those without a diapause.

The Influence of Temperature on the Rate of Development of Post-Diapause Eggs.

(1) *The rate of development at constant temperatures.* The relation between temperature and the rate of development of post-diapause eggs of *A. cruciata* is represented by a sigmoid curve (Fig. 1.). It is seen that between 16·1° C. and 30·5° C. the curves do not conform to the standard equations of Van't Hoff, Arrhenius, Bělehrádek and Krogh, nor to the equation for a second degree parabola. The departure from linearity, as tested by the method of least squares, is significant even when the points for the highest and lowest temperature are excluded from the analysis.

A number of workers on insects have concluded that their data on the relation of temperature and the rate of life processes cannot be explained by any of the several theoretical relationships mentioned above (e.g. Harries, 1939; Powsner, 1935). On purely physiological grounds it is unlikely that the relation between temperature and the rate of life processes is a simple one. Janisch (1933) has formulated the catenary curve to include all temperatures at which development can occur. The rate of development increases with increase in temperature to the point of inflection which Janisch calls the optimum temperature. There does not appear to be any advantage in using one mathematical expression to cover the whole range of temperatures since different processes must be dominant in the determination of the ascending and descending slopes of the curve.

It has not been possible to find a case in the literature in which a temperature developmental curve has been obtained for the post-diapause development of eggs having a diapause so fixed as that of *A. cruciata*. The curves for the post-diapause development of eggs with a less fixed diapause do not differ from those of other insect eggs (Parker, 1930; Zwölfer, 1934). It may be suggested that the influence of diapause on the eggs of *A. cruciata* may extend in some way into the post-diapause period of development; however, the changes which occur at the end of diapause are sudden and well defined. Whereas the development of late diapause eggs is retarded at 30° C. immediate post-diapause eggs have a greatly accelerated rate at this temperature as compared with lower ones.

(2) *The rate of development at alternating temperatures.* It is now generally evident that normal eggs are not stimulated to faster development by alternating temperatures. In eggs with a diapause a preliminary exposure to low temperatures modifies the eggs in such a way that they are able to develop more rapidly when returned to a high temperature (Parker, 1930; Bodine, 1925). In other cases an acceleration has been observed because the low temperature was below the

theoretical threshold of development, but not below the actual threshold, so that some development occurred which was not taken into account by the theoretical straight line relating temperature to the rate of development (Ludwig and Cable, 1933). The non linearity of the relationship may be responsible for a spurious effect of acceleration when variable temperature data are analysed in terms of mean temperatures (Johnson, 1940), or when thermal sums are compared. The stage spent at the particular temperature (Powsner, 1935), or the length of the alternations (Cook, 1927), may be important factors in interpreting some results.

When the eggs of *A. cruciata* were incubated on alternate days at two particular temperatures within the range 16·1° C. to 30·5° C., the rate of development at each temperature was the same as that when the eggs were incubated at these temperatures constantly. Exposure to 32·9° C. retarded development less when eggs were exposed alternately to 32·9° C. and some lower temperature than when they were incubated constantly at 32·9° C. This observation conforms to the conception that the velocity of development at temperatures above the optimum varies with the period of exposure to these temperatures (Bělehrádek, 1935).

SUMMARY.

Experiments have been done on the eggs of *Austroicetes cruciata* Sauss. collected from the field between February and July, 1940.

In the field diapause is eliminated slowly from February (or possibly earlier) onwards; during this time the embryo grows slowly and growth almost ceases during the few weeks preceding katatrepsis.

Some eggs from the lot collected in mid-May when incubated at temperatures between 18·9° C. and 27° C. were the first to hatch in the laboratory. During late diapause, temperatures in this range have some effect in eliminating diapause. Some eggs (8 p.c.) from the lot collected in late May were the first to hatch at 30° C. Ninety-six per cent. of the eggs collected two weeks later hatched at 30° C. This sudden change in the ability of the eggs to hatch at high temperatures is in contrast to the behaviour of the eggs of *Melanoplus differentialis* and some other American grosshoppers.

Normality with respect to development at temperatures above the developmental zero was reached by the entire field population of eggs by mid-June, in the two localities studied. The field population showed great uniformity in this respect. Diapause was eliminated earlier at Orroroo than at Wilmington.

In the field diapause is finally eliminated as katatrepsis is completed, but under artificial conditions katatrepsis may be completed before diapause is completely eliminated.

Two developmental curves for post-diapause eggs are given. They are sigmoidal and do not conform to any simple equation.

Eggs develop slowly at 13·5° C., the rate of development was equivalent to an average rate of 0·9 p.c. per day for short exposures, and 0·7 p.c. per day for longer exposures. One egg hatched at 13·5° C.

The true zero of development is in the vicinity of 8° C.

When post-diapause eggs were incubated on alternate days at two temperatures within the range 16·1° C. to 30·5° C. the rate of development at each temperature was the same as that when the eggs were incubated at these temperatures constantly.

The rates of development of eggs incubated at relative humidities from 70 p.c. to saturation were practically the same.

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A NOTE ON THE OCCURRENCE IN AUSTRALIA OF *CORYNEBACTERIUM EQUI* IN PIGS

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(Accepted for publication 31st January, 1942.)

The name *Corynebacterium equi* was given by Magnusson (1923) to an organism which he showed to be the cause of a purulent broncho-pneumonia in foals. More recently, Magnusson (1938) described the organism in greater detail and stressed the characteristics which differentiated it from *C. pyogenes*. In Australia, Bull (1924) isolated an organism which he considered identical with *C. equi*, from cases of pyaemia in foals while Carne (1927) obtained a similar organism from the kidney of a horse with acute nephritis. Craig and Davies (1940) have isolated *C. equi* in pure culture from the uterine discharges in bovine pyometra.

Holth and Amundsen (1936) examined lesions resembling tuberculosis in pigs and showed that in 59 of 162 cases the lesions were in fact not tuberculosis and in 38 of those lesions acid fast cocco-bacilli were observed. Plum (1938, 1939) obtained similar results in a study of tuberculosis in pigs and from lesions resembling tuberculosis in the submaxillary lymph glands isolated a cocco-bacillus which was shown to be identical with Magnusson's *C. equi*. These findings were confirmed by the work of Bendixon and Jepsen (1938) who showed that their strains of *C. equi* were pathogenic for pigs. Jespersen (1938) described the macroscopic features of lesions caused by "Holth's bacillus" and considered that in the majority of cases it was possible, on macroscopic appearances alone, to distinguish such lesions from those of tuberculosis. Jepsen (1940) recommended the use of tellurite peptone agar as a differential medium for the diagnosis of *C. equi* infection of the lymph glands in the pig. Bendixon and Jepsen (1940) described the histology of lesions produced by *C. equi* and reported further work on the pathogenicity of the organism.

All these observations on *C. equi* infection in the pig have been made in Scandinavian countries, where the infection is apparently quite common, but there appears to be no record of similar findings in any other country. In South Australia, Collins (1939), on the basis of numerous macroscopic examinations, questioned the tuberculous nature of many so-called tuberculous lesions in the lymph glands of the head in pigs. Following these observations, I examined affected lymph glands collected by Mr. Collins at the local abattoir.

MATERIAL AND METHODS.

The specimens examined were the submaxillary lymph glands of pigs which showed no lesions resembling tuberculosis in any other part of the body. In the first cases, the caseous lesions were removed, ground with sterile sand, treated with 5 p.c. H_2SO_4 for 10–30 minutes and neutralized with 4 p.c. NaOH. After centrifuging, the supernatant fluid was discarded and the deposit sown on Petragnini's and Löwenstein's media. In later cases, affected glands were removed and placed in 50 p.c. alcohol for transmission to the laboratory. The lesions were then removed aseptically, ground and smeared on glycerine-egg medium and on Loeffler's medium. By both procedures cultures of a *Corynebacterium* were readily obtained.

DESCRIPTION OF ORGANISM.

Morphology.

The organisms are small, Gram-positive and show a high degree of pleomorphism, which appears to depend to some extent on the medium employed in culture. In shape, the organisms may be small cocci or ovoid in form, short rods with rounded ends, straight, curved or club-shaped.

Staining is uniform, and there are no metachromatic granules. The arrangement also varies. The coccoid forms occur singly, in pairs or in short chains; the rods singly, in pairs often in V formation, or in short chains showing irregular sharp angles, or in clumps. On a few occasions, branching forms were observed. In the lesions, staining by Ziehl-Neelsen's method shows small numbers of tiny, strongly acid-fast coccoid bodies and occasionally small rods with deeply staining granules at each end. In many lesions, however, no organisms were detected on microscopic examination. The organisms are non-motile and non-sporing.

Cultural Characteristics.

Growth occurs readily on ordinary media under aerobic conditions and at temperatures from 18° to 37° C., the optimum temperature being 37° C.

Agar plate. 24 hours at 37° C. Colonies up to 1 mm. diameter, opaque, yellowish, convex, round, smooth, with entire edge, easily emulsified and with marked tendency to coalesce. On further incubation size of colony increases up to 3 mm. diameter.

Agar slope. 24 hours at 37° C. Growth is abundant, confluent, slightly spreading, raised, with smooth surface and entire edge, opaque and yellowish. On prolonged incubation at 22° C. a faint pink pigment is produced by some strains.

Blood agar plate. Growth similar to that on agar plate. No change in medium.

Nutrient broth. 24 hours at 37° C. Uniform, moderate turbidity, with a granular deposit. Some strains produce a thin granular pellicle, which disintegrates easily on shaking. After prolonged incubation, there is a heavy growth which has settled leaving a clear or cloudy supernatant; on swirling the tube, the viscous deposit forms a twisted, ropy mass on a conical base, which is firmly adherent to the bottom of the tube.

Gelatin. 2 weeks at 37° C. Growth moderate, no liquefaction.

Loeffler's medium. 24 hours at 37° C. Rather poor, thin, confluent growth. On further incubation, growth increases and after 3–7 days, a definite pink or yellowish-pink pigment appears. There is no liquefaction of the medium.

Litmus milk. After 7 days at 37° C. no acid or clot is produced, but the medium may change to dull yellowish-brown colour.

Fermentation. In the fermentation tests, good growth is obtained in 1 p.c. peptone water containing 1 p.c. of the carbohydrate to be tested. No acid or gas is produced in glucose, lactose, maltose, sucrose, galactose, mannite or dextrin after 8 days at 37° C.

Antigenic Structure.

This has not been studied.

DISCUSSION.

Bergey (1939) lists *C. equi* Magnusson in a group of 30 species, which are not included in his classification on the ground that they have been incompletely described and may be identical with recognized species. The somewhat scanty information available in many cases renders classification extremely difficult, and it seems highly probable that more detailed examination, and particularly serological study, would enable many of these organisms to be suitably classified. In view of this uncertainty, it is with some reserve that the organism described above is termed *Corynebacterium equi*; however, it conforms in all essential details with the original description given by Magnusson, and has been isolated from lesions apparently identical with those described by Scandinavian workers. The obvious economic importance of the organism in its relation to lesions resembling tuberculosis in the pig, has rendered it desirable to record its occurrence in Australia and to draw attention to this source of economic wastage.

SUMMARY.

From lesions resembling tuberculosis in the lymph glands of pigs, an organism, apparently identical with *Corynebacterium equi* Magnusson, has been isolated.

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ADDENDUM.

Since this manuscript was submitted for publication, my attention has been drawn to the work of D. W. Bruner and P. R. Edwards (1941), (Kentucky Agr. Exp. Stn., Bull. 414) on the classification of *C. equi*. They quote the view of W. H. Feldman, H. E. Moses and A. G. Karlson (1940), (Cornell Vet., 30, p. 465) that in America *C. equi* may be the cause of lesions resembling tuberculosis in swine. Bruner and Edwards examined serologically a number of cultures derived from several countries and from several species of animals. The cultures included strains isolated from lesions in the lymph glands of pigs. The organism was shown to possess species—specific antigens, which were demonstrated by complement fixation tests. These antigens were not apparent in other species of diphtheroids used for comparison. This work suggests that by serological examination it should be possible to distinguish *C. equi* from other members of the genus which show similar morphological and cultural characteristics.

EXPERIMENTAL POLIOMYELITIS

2. FAILURE TO TRANSMIT THE DISEASE TO JERBOA MICE (*NOTOMYS MITCHELLI*), RATS (*RATTUS NORVEGICUS*) AND PHALANGERS (*TRICHOSURUS VULPECULA*)

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In 1939 Armstrong announced the transmission of the Lansing strain of poliomyelitis virus to Eastern cotton-rats *(Sigmodon hispidus hispidus)*, and in the following year Jungeblut and Sanders (1940) succeeded in adapting the SK New Haven strain to the same animal. Toomey and Takacs (1940a) confirmed Armstrong's findings with the Lansing strain, but obtained only negative results with nine other strains.

On the other hand, Kessel and Stimpert (1940), and Howitt and Van Herrick (1941), using a considerable number of strains, were unable to transmit poliomyelitis to several wild species of rats and mice. Similar results were reported by Hammon (1940), using the "spreading factor" of Duran-Reynals.

Recently, Toomey and Takacs (1940b, 1941a, 1941b, 1941c), by giving preliminary or concomitant inoculations of bacteria-free filtrates of ten day glucose-broth cultures of certain organisms of the genus *Bacterium*, succeeded in adapting Flexner's M.V. and Philadelphia strains of poliomyelitis virus to cotton-rats.

In view of the above findings it seemed of interest to determine whether an Australian strain of poliomyelitis virus was transmissible to rats, to a marsupial (phalanger) and to a placental mammal (Jerboa mouse).

MATERIALS AND METHODS.

All monkeys used were *M. mulatta*. The rats were derived from a laboratory strain of the common brown rat. Jerboa (jumping) mice and phalangers (bushy-tailed opossums) were obtained by trapping. Animals not sacrificed in the course of the experiments were observed for at least one month following inoculation.

The organism selected from the genus *Bacterium* was a local strain of *B. coli* (B4). It was grown in glucose-broth for 11 days. The culture was then centrifuged and the supernatant filtered through a Berkefeld N filter. The pH. of the filtrate was approximately 6.

The virus was derived from a mixture of two local strains, isolated in Victoria and Tasmania, respectively, during the major epidemic of 1937–8. Spinal cords were preserved in 50 p.c. buffered glycerol at — 10° C. Suspensions (10–20 p.c.) were prepared by grinding with sterile washed sand and normal saline. In some instances *B. coli* filtrate was substituted for the saline. After centrifuging at 3,500 r.p.m. for 5–10 minutes, the supernatant was used as the inoculum. Suspensions of the brains of various passage animals were prepared similarly.

The dose of inoculum was 0·025–0·05 c.c. for Jerboa mice, 0·05 c.c. for rats, 0·5 c.c. for opossums and 1 c.c. for monkeys. Except where otherwise stated injections were given by the intracerebral route.

A. Experiments with Jerboa mice. 1. Three Jerboa mice and a monkey (S.96) were inoculated with poliomyelitis virus; the last became paralysed on the 8th day. Two of the mice were killed on the 7th day and a suspension of their brains was injected into 3 further mice. In all, five passages at intervals of 7 days were carried out, one mouse of each passage being retained for observation. None of the animals developed paralysis. One of the 5th passage mice died on the 6th day; histological examination, however, proved negative. Two monkeys, S.114 and S.115 inoculated intracerebrally and intramuscularly (10 c.c.) with a brain suspension from the 3rd passage in Jerboa mice remained free from symptoms. Subsequent intracerebral and intramuscular injection of S.114 with the original poliomyelitis virus led to paralysis on the 12th day.

[1] Working with the aid of a grant from the National Health and Medical Research Council.

2. Two Jerboa mice were inoculated with poliomyelitis virus on three successive days. Both remained well.

3. Two Jerboa mice were given *B. coli* filtrate on three successive days; one acted as a control while on the 4th day the other was injected with poliomyelitis virus. In addition another mouse was inoculated with poliomyelitis virus prepared with *B. coli* filtrate instead of saline. None of the three animals developed paralysis.

B. Experiments with rats. At each passage the rats were divided into three groups. The first group (1 animal) was given *B. coli* filtrate on three successive days in order to eliminate the possibility of activation of a virus already present. The two rats comprising the second group were inoculated with *B. coli* filtrate similarly to the first group; on the fourth day they were injected with poliomyelitis virus. Finally the third group (1 animal) was inoculated with the virus alone.

At the end of 7 days one animal of each group was killed, and suspensions of their respective brains were injected into further rats, which had received preliminary inoculations similar to those of the first three groups. Three passages were carried out. None of the rats, including those allowed to survive each time from Group 2, developed paralysis. After three passages equal parts of brain suspensions from Groups 2 and 3 were inoculated in monkey M.982 with negative results.

Experiments with opossums. The opossums were divided into two groups each containing two animals. The first group was given an injection of poliomyelitis virus prepared with saline; in the second group *B. coli* filtrate was substituted for the saline. One animal of each group was kept for observation; the second was killed and a suspension of its brain (prepared as before) was inoculated into two further animals. After three passages brain suspensions from each group were injected into monkey M.983 which remained well. In the course of the experiments two opossums died from non-specific causes. Two others became apathetic and were killed. Histological examination of the brains of these two animals proved negative for one of them, while the other revealed encephalitozoon infection.

DISCUSSION.

In the transmission of poliomyelitis to cotton rats three factors appear to be of importance: viz. (1) the strain of virus, (2) the rapid passage of the virus from animal to animal without waiting for the development of symptoms (Jungeblut and Sanders), and (3) the breaking-down of neural resistance with the aid of filtrates of glucose-broth cultures of organisms of the coli-typhoid-paratyphoid group (Toomey and Takacs). Despite attention to the second and third factors, the results of the present investigation are uniformly negative. Nevertheless, on account of the high cost of monkeys as experimental animals, and in view of the fact that so far cotton-rats have proved susceptible only to a few strains of poliomyelitis virus, and then only by the intracerebral route, every opportunity should be taken to test the susceptibility of other animals to the disease.

SUMMARY.

Negative results were obtained in attempts to transmit an Australian strain of poliomyelitis virus to Jerboa mice, rats and phalangers.

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PHARMACOLOGICALLY ACTIVE SUBSTANCES IN SERUM

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Some reports, based on the one hand on clinical experience (Aldrich *et al.*, 1938; Strumia, Wagner and Monaghan, 1940; Black, 1940) and on the other hand on animal experiments (Buttle, Kekwick and Schweitzer, 1940; Levinson, Neuwelt and Necheles, 1940) have suggested that human serum is inferior to plasma for intravenous injection in man.

Nevertheless, considerable numbers of serum transfusions have been given both to man and animals without injurious effects (Bond and Wright, 1938; Wright, Bond and Hughes, 1938; Clegg and Dible, 1940; Magladery, Solandt and Best, 1940, Best and Solandt, 1940a, Hill, McMichael and Sharpey Schafer, 1940); and Best and Solandt (1940b) state quite definitely that serum is in no way inferior to plasma.

On *a priori* grounds, reactions following the injection of large doses of human serum might be attributable to (1) the presence of thrombin in freshly prepared serum, (2) bacterial contamination with the resultant formation of pyrogens, (3) a high agglutinin titre of the serum or (4) the presence of pharmacologically active substances liberated in the process of clotting. The first possibility can be excluded by storage for a week or more thus ensuring the disappearance of thrombin and the second by collection of the blood with strict asepsis, by the addition to the plasma of a bacteriostatic agent (merthiolate 1 : 10,000) and by early filtration of the serum. The third possibility can be avoided by a process of pooling recently described by Jakobowicz and Bryce (1941).

The present paper is concerned with the pharmacologically active substances in serum, the methods by which their presence may be determined and the conditions most suitable for the preparation of serum containing them in minimal amounts. The presence of such active substances in serum has long been known. The earlier work is reviewed by Janeway, Richardson and Park (1918) and the more recent papers by Amberson (1937). Vasoconstrictor and vasodilator substances, and substances having a stimulant effect on plain muscle have been described. As early as 1869 Ludwig and Schmidt (1869) found that defibrinated blood could be perfused only with difficulty through the muscle of the dog. Serum was found to have a stimulant effect upon isolated arterial preparations by Meyer (1906) who attributed it to the presence of adrenalin. O'Connor (1912) showed that this was not the case and confirmed the observation of Stevens and Lee (1884) that the active substances were formed during clotting. Janeway, Richardson and Park (1918) provided evidence that the muscle stimulant substances in serum were formed from the platelets.

The phenomenon described by Brodie (1900)—a fall of blood pressure in the cat produced by the intravenous injection of homologous and heterologous sera—has been further studied.

METHODS.

Sterile human blood serum from fasting subjects was obtained by a variety of methods. Altogether about 110 samples were tested. In some cases blood was collected without the addition of an anticoagulant and was allowed to clot, standing at room temperature for six hours the serum being removed after a further 18 hours at 0–2° C. Serum was also prepared by the addition

[1] This work was aided by a grant from the National Health and Medical Research Council.

of calcium chloride to oxalate plasma (Morgan, 1927), separated from the formed elements by (1) gravity (0–2° C. for 24 hours) or (2) in a modified cream separator or (3) by centrifugation. Some of the sera prepared in the last named way came from the Commonwealth Serum Laboratories, Melbourne. Citrated plasma was prepared by the addition of 4 p.c. sodium citrate to blood (1: 10) and in a few experiments such plasma was converted into serum by the addition of 20 p.c. calcium chloride (1: 80). By differential centrifuging (see under results) plasma could be obtained free from leucocytes but rich in platelets. Centrifugation at 4,500 r.p.m. for 20 minutes usually reduced the platelet count to 1,000 or 2,000 per c.mm. Such plasma has for convenience been termed "platelet-free" plasma. Serum was usually kept in the refrigerator at 0–2° C.

Some samples of serum were dried for us by Mr. Holden by the method of Flosdorf and Mudd (1935). Serum for drying by the method of Hardy and Gardiner (1910) was first concentrated to half its volume in cellophane tubes placed in a draught of dry warm air. The concentrated serum was then poured into 10 volumes of a mixture of 7 parts ethyl alcohol (99·5 p.c.) and three parts anhydrous ether at — 12° to — 14° C. The precipitate after standing three hours in the cold was washed aseptically with 10 volumes of ether by a method elsewhere described (Bick) and dried *in vacuo* over sulphuric acid. Ultrafiltrates of serum were in the early experiments prepared by the method of Adair (1925) using the modifications described by Kellaway (1928). In the later experiments ultrafiltration was carried out through cellophane membranes supported in a Seitz filter, the serum on one side being subjected to a pressure of 60 mm. of mercury, and the filtrate on the other side being led usually into an evacuated flask. Dialysis was carried out in cellophane tubes against running water at room temperature or against 12 hourly changes of distilled water in the refrigerator (20 c.c. serum against 2 litres). The preparation of extracts of serum and platelets is described with the results.

Various preparations were used to test for the presence of pharmacologically active substances in serum: the isolated jejunum of the guinea-pig, the heart of the atropinized guinea-pig (Drury, Lutwak-Mann and Solandt (1938)), the isolated uterus of the virgin rat, the isolated rabbit's ear and the ox carotid strip. Of these, only the last two require any special description.

The rabbit's ear. The animal was anaesthetized with ether and a blunt-ended hypodermic needle, grooved near its tip, was introduced distally into the largest artery at the base of the ear. The ear was amputated and the blood in its vessels rapidly syringed out with Ringer's solution through the needle cannula. The preparation was then placed on an inclined glass plate and connected by the butt of a all-glass syringe to the perfusion apparatus. Perfusion was carried out with a head of pressure of 100–150 cm. of water. The Ringer used contained sodium chloride 0·9 p.c., potassium chloride 0·042 p.c., calcium chloride 0·012 p.c., sodium bicarbonate 0·1 p.c. Oxygen containing 3 p.c. CO_2 was bubbled through the reservoir and the Ringer's solution was thus maintained at pH 7·4. The reservoir was placed in a thermostat and the Ringer's solution flowed through a Gunn's perfusion apparatus whose water jacket was supplied from the thermostat. The perfusion fluid was thus maintained at 37° C. despite changes in the rate of flow. The ear was kept warm by a lamp placed near it. The rate of flow through the ear was estimated by counting the drops per minute collected from the glass plate. Serum was tested by injecting it with a syringe and needle into a rubber connection proximal to the cannula.

The ox carotid. For this preparation a modification of the technique described by Meyer (1906) was used. Ox carotid arteries were obtained from animals freshly killed at the abattoirs. They were at once washed with ice-cold Ringer's solution and, immersed in this solution, were transported to the laboratory and stored at 0° to 2° C. Spiral strips of wall 8 mm. wide and 4 cm. long were cut from the middle portion of the artery. A cotton thread for attachment to a frictionless recording lever was affixed to one end by a mattress suture and a small loop of thread was similarly attached to the other end. The preparation was suspended at 37° C. in an isolated organ bath of 10 c.c. volume, the needle carrying the oxygen passing through the thread loop. The preparation was stretched for 20 minutes by a tension of 80 gm. which was reduced for testing to 30 gm. as described by Janeway, Richardson and Park (1918). For testing, the sera were left in contact with the preparation for 1 to 1½ minutes, at the end of which time the Ringer's solution in the bath was changed.

Experiments on the Brodie effect. Cats, rabbits and dogs were anaesthetized with chloralose 10 c.c. per kg. of 1 p.c. solution being injected intravenously. The dogs were premedicated with morphine 10–15 mg. per kg. The systemic blood pressure was recorded from a carotid artery and the pulmonary arterial pressure was recorded by the method of Kellaway and LeMessurier (1936). The pulmonary venous pressure was recorded by a Brodie tambour connected through a "U" tube to a cannula in a pulmonary vein; the cannula and "U" tube contained citrated saline to which some heparin was added.

Perfusion of the heart of the cat was carried out as described by Feldberg and Kellaway (1938) using Gunn's modification of Langendorff's method. In some experiments a lever attached by a thread to the apex recorded the heart movements on a smoked drum.

For perfusion of the cats' mesentery the animals were anaesthetized with ether, a glass cannula tied into the superior mesenteric artery and the mesentery perfused with Ringer's solution. The mesentery was separated from its attachment to a length of small bowel by dividing

the peritoneum on either side of the intestine. The blood vessels supplying the remainder of the small and large bowels were tied and divided and the isolated mesentery so obtained was suspended from the cannula and surrounded by a water jacket at 37° C. Perfusion and injection were carried out as described for the rabbit's ear.

RESULTS.

Part 1. The Muscle Stimulating and Vasoconstrictor Substances in Serum.

1. *General effects of the muscle stimulant substances in serum.* For the investigation of these active substances the isolated jejunum of the guinea-pig and the ox carotid strip were the most suitable preparations and gave generally parallel results.

(a) *The jejunum of the guinea-pig.* Samples of serum varying in age from 12 hours to several weeks obtained by clotting whole blood, caused contraction of the jejunum of the guinea-pig (in an organ bath of 10 c.c.) in doses of from 1·0 to 0·2 c.c. after latencies of from 4 to 10 seconds. The magnitude of the responses varied in different preparations and even in the same preparation; successive doses at 10 to 20 minute intervals giving at first increased but sometimes after an hour or more, decreased or irregular responses. In most preparations it was possible to get good comparisons of the activity of sera, the tests being made at 10 minute intervals. The sera were left in contact with the gut for 30 seconds before washing out; relaxation was rapid although sometimes interrupted by spontaneous contractions. After each contraction the excitability of the gut as indicated by its response to histamine was often decreased for a short period and then increased. A dried sample of serum prepared from clotted whole blood, reconstituted immediately before testing, was used as a standard. Numerous samples of fresh serum from clotted whole blood showed activity varying within 30 p.c. above or below that of the standard. Fig. 1 shows the failure of the isolated gut to respond to 1 c.c. of whole blood at B and its response at S to 0·7 c.c. of fresh serum obtained from the same sample of blood. These responses had a latency of 5 seconds.

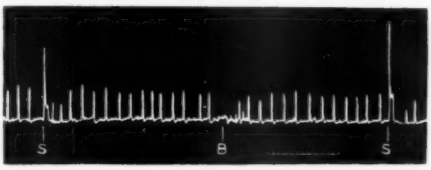

Fig. 1. Responses of a piece of isolated jejunum from a guinea-pig to 0·7 c.c. of serum from clotted human blood at S. At B, 1 c.c. of whole blood was removed from a vein and immediately tested without anticoagulant. The unlettered responses as to 0·1γ histamine. Time in half minutes.

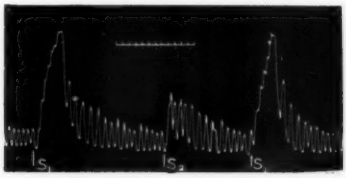

Fig. 2. Response of a horn of the isolated uterus of a virgin rat. At S_1, 1·0 c.c. of the standard reconstituted dried serum and at S_2, 1·0 c.c. of a sample of reconstituted dried serum prepared from pooled centrifuged human plasma. Time in half-minutes.

(b) *The uterus of the virgin rat.* This preparation is less sensitive than the isolated jejunum of the guinea-pig to the active substances in serum. It also varies a good deal in sensitivity and sometimes responded to serum which was otherwise inactive. Usually an active serum in a dose of 0·5 to 1·0 c.c. added to the 10 c.c. organ bath causes a strong contraction with inhibition of rhythm after a latent period of 10 to 15 seconds. When after 2 to 3 minutes the Ringer's solution in the bath is changed the rhythm returns, the muscle gradually relaxing in about 5 minutes.

Fig. 2 shows the type of response obtained and also illustrates a general finding that serum prepared from whole blood is generally more active than serum from centrifuged plasma. A sample of 1 c.c. of the standard reconstituted serum at S_1 gave a much larger response than the same dose of serum prepared from centrifuged plasma at S_2.

(c) *The ox carotid.* This preparation is very suitable for investigating vasoconstrictor effects. It contracts after a latent period of 3 to 20 seconds to doses of 0·5 to 2 c.c. of human serum prepared from whole blood. Usually the serum was left in contact for 60 to 90 seconds. Fig. 4 illustrates the type of response obtained. Repeated tests could be made in these preparations which often remained fairly constant in sensitivity for about two hours. Relaxation takes place after each response in from 5 to 20 minutes but after some hours at 37° C. the preparations fail to relax completely and though extremely sensitive become unreliable. Centrifuged oxalate

or citrated plasma was usually without activity as was also serum prepared from it. Whole blood without anticoagulant or citrated gravity plasma while usually without activity sometimes produced a response after a latency of 60 seconds or longer, presumably due to the clotting process occurring in the organ bath.

(d) *The rabbit's ear.* This preparation varied somewhat in sensitivity. Usually the standard serum in a dose of 0·5 to 1 c.c. within a minute after injection caused vasoconstriction which persisted for from 5 to 45 minutes. In a few preparations a dose of 1 c.c. had only a trivial vasoconstrictor effect. The sensitivity of the preparation increased with successive doses. Sera were compared by administering them in alternate doses. Generally, as with the other preparations, sera prepared by clotting the plasma were less actively vasoconstrictor than those prepared by clotting whole blood. All the sera tested had some activity and it is possible that this may have been attributable in part to the temporary increase in the viscosity of the perfusion fluid and not wholly to active substances in them.

2. *Comparison of the muscle stimulant activity of sera prepared by various methods.*

(a) *Sera prepared by clotting whole blood and by clotting plasma.* As already mentioned serum prepared by clotting centrifuged plasma is generally less active than serum from whole blood. Sera prepared from centrifuged plasma by the method described by Morgan (1927), using a modification which he has recently adopted, were regularly found to be less active than the standard. The ox carotid nearly always failed to respond to these sera in doses of 2 c.c. and the guinea-pig jejunum gave only a feeble response or none at all to doses of 1 c.c.

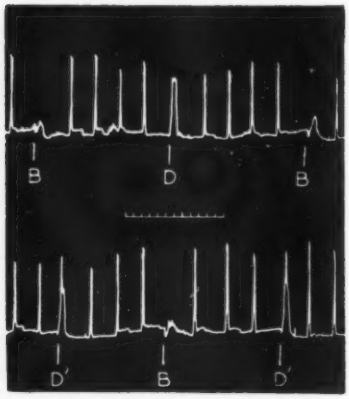

Fig. 3. Responses of a piece of isolated jejunum of a guinea-pig to 1 c.c. of serum prepared from plasma (at B) and to 1 c.c. of serum from whole blood (at D). In the lower tracing the same sera after adjustment of the K and Ca content were tested. The unlettered contractions are to 0·1γ histamine. Time in half-minutes.

In most of the experiments two or more different samples of serum were prepared from the same blood, e.g. from whole blood and from centrifuged oxalate plasma. The former were about equal in activity to the standard, whereas the latter were almost devoid of activity in doses of 1 c.c. Fig. 3 (upper tracing) shows the response of a piece of jejunum to 1 c.c. of serum from whole blood at D and of serum from centrifuged plasma at B. It was thought possible, however, that excess of potassium or calcium in the sera prepared by Morgan's method might mask the response to the smooth muscle-stimulating substances. Saline in doses of 1 c.c. containing 50–200 mg. p.c. of K ion or 20–100 mg. p.c. of Ca ion when administered to the gut produced relaxation, preparations varying in sensitivity to these ions. Accordingly in some experiments the ionic content of the sera undergoing comparison was adjusted either by adding potassium oxalate and calcium chloride in calculated amount, to the serum from whole blood, or by estimating the ionic content of the sera and adding potassium chloride or calcium chloride or both so as to equalize the ionic content of the sera whose activity was to be compared.

Fig. 3 (lower tracing) shows the responses to the same sera after the addition of potassium chloride to the serum from whole blood, the difference in Ca ion content being too small to need correction. We were not able to account for the difference in activity of the sera by the masking effect of excess of Ca or K ion.

(b) *Variations in activity of sera prepared by clotting plasma.* The activity of serum depends upon the number of platelets present in the plasma at the time of clotting. This was indicated by the kind of experiments described below. In one type of experiment a portion of freshly drawn blood was allowed to clot and the serum removed after 24 hours. Another portion was oxalated and allowed to settle by gravity for 24 hours, the resultant gravity plasma being divided into two parts one of which was clotted by the addition of calcium chloride, the other being centrifuged (4,500 r.p.m. for 20 minutes) and then clotted in the same manner. In one such experiment the platelet count of the "gravity plasma" was 270,000 per c.mm. and that of the centrifuged plasma 6,500 per c.mm. Serum prepared from the former had about 65 p.c. of the activity of the standard whereas serum prepared from the latter had practically no activity. Serum prepared from the whole blood had 75 p.c. of the activity of the standard. Fig. 4 shows

the results of tests on the ox carotid and Fig. 5 those on the isolated jejunum of the guinea-pig in a similarly planned experiment. In Fig 4 at D are the responses of the ox carotid to 2 c.c. of serum prepared from whole blood and at A that to 2 c.c. of serum from 24 hour gravity plasma containing 310,000 platelets per c.mm. The same dose of serum from plasma prepared by centrifugation of whole blood and containing 3,000 platelets per c.mm. at B and of serum from centrifuged gravity plasma (2,200 platelets per c.mm.) at C gave no response. In Fig. 5 are shown the responses of the guinea-pig jejunum to 1 c.c. of blood from whole blood at D and to 1 c.c. of serum from gravity plasma at A. At C, 1 c.c. of serum from centrifuged gravity plasma failed to cause any response. It can be seen that whereas serum from centrifuged gravity plasma is almost devoid of activity that from gravity plasma is nearly as active as that obtained from whole blood. Altogether 12 such experiments were performed.

Fig. 4. Response of isolated strip of ox carotid to 2 c.c. doses of sera prepared from whole blood and from corresponding plasma. Time in half-minutes. Details in text.

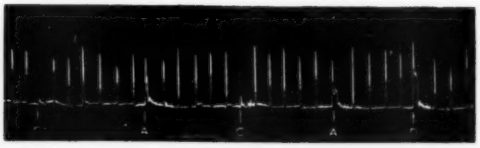

Fig. 5. Response of a piece of isolated jejunum of the guinea-pig to the same sera tested in Fig. 4. The unlettered responses are to 0·1γ histamine. Time in half-minutes. Details in text.

In other experiments by differential centrifugation of gravity plasma (as described in the section on platelet extracts) platelet-rich plasma, free from other formed elements, could be obtained. Serum prepared from such plasma was quite active on the ox carotid and jejunum of the guinea-pig whereas that obtained from the corresponding platelet-free plasma was without activity in doses of 2 and 1 c.c. respectively (see Fig. 9).

"Cream-separated" sera were also tested. The platelet count of cream-separated plasma was higher (50–150 × 10^3 per c.mm.) than well centrifuged plasma, and serum prepared from the former was correspondingly not completely devoid of activity. Quantitatively, however, such sera were usually about ¼ or ⅓rd as active as the standard.

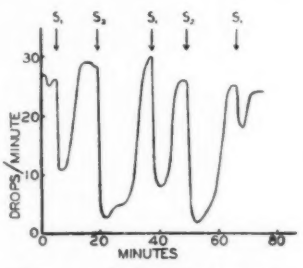

Fig. 6. Graph showing the effect on the outflow from the rabbit's ear of the injection of 1 c.c. doses of human serum from centrifuged plasma at S_1 and from whole blood at S_2. Details in text.

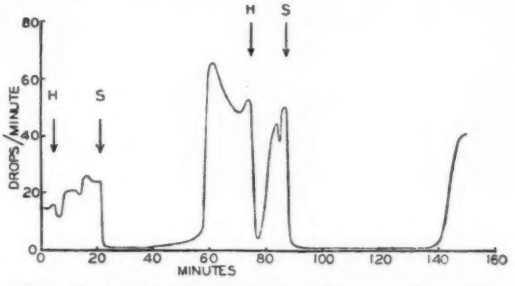

Fig. 7. Graph illustrating the effect on the outflow from the rabbit's ear of the injection of 1 c.c. doses of reconstituted Hardyized serum at H and the same dose of the reconstituted standard serum at S. Details in text.

Serum prepared from plasma containing few or no platelets caused some diminution in outflow from the rabbit's ear but this effect was much less than that of serum prepared by clotting whole blood. This is illustrated in Fig. 6. At S_1 a 1 c.c. dose of serum from centrifuged oxalate plasma containing 2,000 platelets per c.mm. gave a less extensive and less prolonged constriction than an equal dose of serum from the same whole blood.

(c) *Experiments with serum from whole blood dried by Hardy's method.* This was very readily and completely soluble in water or saline solution and was dissolved for use in 1 part water and 2 parts Ringer's or Tyrode solution.

It had no activity on the jejunum in doses equivalent to 1 c.c. of serum or on the ox carotid in doses of 2 c.c. On the other hand the saline extracts of lipins and other substances obtained in concentrated form by evaporation of the alcohol-ether mixture used to precipitate the serum proteins gave responses on the jejunum in doses equivalent to 0·2 c.c. of the original serum. The contraction so produced appeared and relaxed more slowly than that of serum and sometimes resembled the response to slow-reacting substance produced by the action of snake venom on egg yolk (Feldberg, Holden and Kellaway, 1938). There was however no diminution of the response to repeated doses. The extract in doses equivalent to 20 c.c. of serum had no action on the carotid strip.

On the isolated rabbit's ear, Hardyized serum caused only a transient diminution of outflow. Fig. 7 illustrates this effect and also shows the increase in sensitivity of the ear with repeated doses. Hardyized serum equivalent to 1 c.c. (at H) gave short-lasting effects the second of which was greatly increased, and 1 c.c. of standard serum prepared from clotted whole blood at S gave prolonged vasoconstriction.

3. Experiments with platelet extracts.

The experiments described above like those of earlier workers (Stewart and Zucker (1913), le Sourd and Pagniez (1914), Janeway, Richardson and Park (1918) and Freund (1920)) point to the platelets as the source of the muscle-stimulant activity in serum. These workers have shown that extracts of platelets have a stimulant action upon plain muscle but their experiments do not clearly indicate any quantitative relationship between the activity of such extracts and that of corresponding amounts of serum.

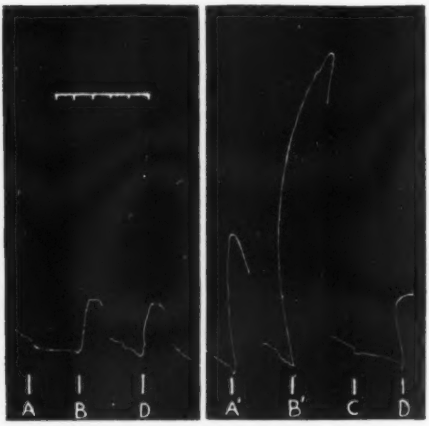

Fig. 8. Tests on the ox carotid of unboiled platelet extracts at A, A_1, B and B_1, of serum from platelet-free plasma at C and from platelet-rich plasma at D. The injections at A, B, C and D correspond to 2 c.c. of plasma. Time in minutes. Other details in text.

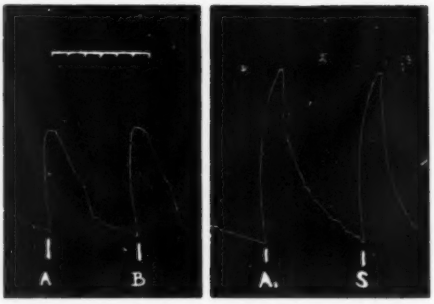

Fig. 9. Tests on the ox carotid of 0·4 c.c. doses of boiled extract of washed platelets at A and of boiled extract of unwashed platelets at B. On a new piece of carotid the activity of 0·4 c.c. of the washed platelet extract at A_1 is compared with that of a corresponding dose (2 c.c.) of serum prepared from the original platelet containing plasma at S. Time in minutes.

To show this quantitative relationship six experiments of the following kind were performed. Gravity plasma, oxalated or citrated, was centrifuged four times at 2,000 r.p.m. for one minute to remove the leucocytes discarding the sediment after each centrifugation. The supernatant fluid after determination of its platelet content was divided into three equal portions. The first was clotted by the addition of $CaCl_2$ and serum was so obtained; the second and third were centrifuged at 4,500 r.p.m. for 15 minutes to throw down the platelets. The sediment from the second twice washed with physiological saline solution, and the unwashed sediment together with about one-fiftieth of the original volume of plasma from the third were used to make saline platelet extracts by grinding with finely divided quartz. To each calcium chloride was added in calculated amount. The extract containing the unwashed platelets clotted and the active fluid was expressed from the clot. Fig. 8 shows the test of the extracts in such an experiment on the isolated ox carotid. The plasma used contained 193,000 platelets per c.mm. and 1 c.c. of each

of the platelet extracts corresponded to 10 c.c. of plasma. At A and A′ 0·2 and 1 c.c. of extract of washed platelets, at B and B′ the same doses of extract of unwashed platelets, at C 2·0 c.c. of serum prepared from the platelet-free plasma and at D 2 c.c. of serum prepared by clotting platelet-containing plasma were tested. The unwashed platelets for a given quantity of plasma yielded an extract whose activity was equivalent to a corresponding amount of serum prepared from the original platelet-containing plasma. The washed platelet extract was sometimes less active, as in this experiment. Washed platelet extracts, however, were usually increased in activity by bringing to the boil and after this treatment they were equal in activity to the corresponding boiled extract of unwashed platelets. Fig. 9 shows the tests on the ox carotid of boiled extracts of another sample of platelets, 1 c.c. corresponding to 5 c.c. of plasma. The responses to 0·8 c.c. of boiled extract of washed platelets are shown at A and that to the same dose of boiled extract of unwashed platelets at B. With a new piece of carotid 0·4 c.c. of a saline extract of washed platelets gave the response shown at A_1 and that of 2 c.c. of corresponding serum at S. None of these responses was maximal. Saline platelet extracts had also a stimulating action on the guinea-pig jejunum and caused vasoconstriction in the isolated rabbit's ear. Saline extracts were also made from fresh platelets dried *in vacuo* over sulphuric acid and from dried platelets after preliminary extraction with methyl alcohol. These extracts possessed some activity but in general not as much as extracts of freshly centrifuged platelets. The methyl alcohol extracts possessed no activity.

4. The occasional activity of plasma.

Occasionally plasma rich in platelets acquired activity after storage (0–2° C.) for a week or more, the corresponding platelet-free plasma remaining inactive. This was not surprising because the experiments described above suggest that the active substance liberated in the clotting process is already preformed in the platelets. Indeed it was possible to activate platelet-rich plasma by mechanical shaking with glass beads to bring about platelet breakdown. Such plasma after shaking for 30–60 minutes was in three experiments found to be as active quantitatively as serum prepared from it, and in two other experiments it was much more active.

Fig. 10 shows the record of such an experiment. At G is shown the response of the isolated ox carotid strip to 1 c.c. of unshaken citrated gravity plasma. At G_1, 1 c.c. of the same plasma was given after shaking with glass beads for half an-hour. At S, 1 c.c. of serum prepared by adding calcium chloride in calculated amount to the gravity plasma was injected, and at C 1 c.c. of shaken centrifuged plasma.

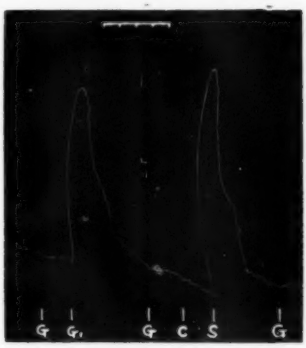

Fig. 10. Responses of the ox carotid to 1 c.c. doses of citrated gravity plasma at G, shaken gravity plasma at G_1, shaken centrifuged plasma at C, and serum from gravity plasma at S. Time in minutes. Details in text.

5. Some properties of the muscle-stimulating substances.

Effect of storage. Drying of serum by the method of Flosdorf and Mudd is without effect on its activity. As already stated such a dried serum was used as a standard. By drying a sample of serum and after the lapse of time comparing its activity with that of the original liquid serum the effect of storage could be determined. Such experiments showed that sterile serum with few exceptions lost little if any of its activity after storage for 3 months either at 0–2° C. or at room temperature. On the other hand platelet extracts and filtrates of serum (see below) lost nearly all their activity within a week or less.

Effect of temperature. Many platelet extracts were brought to the boil in the course of preparation usually with an increase in their activity. In several experiments serum (twice diluted with saline) was boiled and the proteins precipitated by the addition of N/10 hydrochloric acid. The filtrate from such a mixture was neutralized with sodium hydroxide (litmus indicator) and was found to have retained its stimulating action on the ox carotid and the jejunum. More prolonged boiling (20 minutes) occasionally diminished the activity.

Solubility. Extracts of dried serum in ethyl or methyl alcohol or ether were prepared by extracting the equivalent of 10–30 c.c. with the solvent for 24 hours, filtering, washing the residue with solvent, evaporating the filtrate and washings (either in a water bath or *in vacuo* at room temperature) and emulsifying the residue so obtained with saline. Such extracts were without activity on the ox carotid (even sometimes in doses equivalent to 20 c.c. of serum) but the alcoholic extracts had usually some action on the isolated jejunum of the guinea-pig. Such responses were however of longer latency, slower in development and relaxation and were much

smaller in magnitude than those produced by corresponding amounts of serum. The reconstituted dried serum after extraction had retained practically all its activity for the gut and carotid strip.

Ultrafiltration and dialysis. Twenty-four hours dialysis through cellophane membranes (either in running tap water or against 12 hourly changes of distilled water, 20 c.c. against 2 litres) reduced the activity of the serum to ½ or ⅓ of the original value and 48 hours dialysis usually reduced the activity to less than ¼.

Fig. 17 upper tracing shows the effect on the ox carotid of 2 c.c. of serum at S and of a corresponding volume of dialysed serum at D. This sample had lost practically all its activity after 24 hours dialysis through cellophane in running tap water at room temperature. Dialysis through celloidin membranes also reduced the activity of the serum.

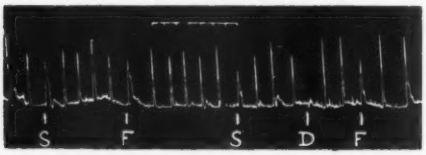

Fig. 11. Responses of the jejunum of the guinea-pig to a 1 c.c. dose of serum at S and corresponding doses of ultrafiltrate of serum at F and a 24 hour dialysate of serum at D. The unlettered responses are to 0·1γ histamine. Time in minutes. Details in text.

Difficulty was experienced in obtaining active ultrafiltrates using the method of Adair with celloidin membranes. In not one of six experiments could an active ultrafiltrate be obtained but active ultrafiltrates could regularly be obtained through cellophane membrane in a Seitz filter. In either case the membranes were permeable to erythrodextrin but not to soluble starch. Failure to obtain ultrafiltrates with celloidin membranes is difficult to explain and perhaps may be attributable to adsorption in the membrane. Ultrafiltrates were however very unstable, losing activity in two days or less and the exposure to the atmosphere of a relatively large surface of filtrate issuing from the celloidin thimble may have contributed to its activity. Nevertheless filtrates through cellophane were active even when not collected *in vacuo*.

Fig. 11 shows the responses of the jejunum of the guinea-pig to a 1 c.c. dose of serum at S and corresponding doses of an ultrafiltrate of the serum at F and the serum after 24 hour dialysis against running water at D. Twenty-four hours later the ultrafiltrate had lost more than half of its activity.

Part 2. The Alleged Presence of Adenyl Compounds in Serum.

Among the pharmacologically active substances said to be present in serum or defibrinated blood are adenyl compounds (Zipf, 1931). Tests of a number of sera (12 hours or more after preparation) in 1 c.c. doses by the method of Drury, Lutwak-Mann and Solandt (1938) failed to show the presence of active adenyl compounds. The serum in this dose usually caused an increase in the amplitude of the auricular contraction and a temporary decrease in the sensitivity of the preparation to adenyl compounds, recovery occurring in from 15 to 20 minutes. Nevertheless, when adenosine was added to serum the mixture tested immediately had the same activity as a similar concentration of adenosine in saline. Serum therefore does not mask the activity of this adenyl compound.

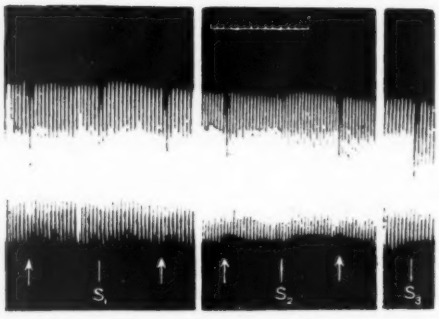

Fig. 12. Record from the right auricle of an atropinized guinea-pig. Time in half minutes. Details in text.

Failure to demonstrate active adenyl compounds in stored sera is not surprising because human sera regularly contain an enzyme which inactivates these compounds (Drury, Lutwak-Mann and Solandt, 1938). Serum to which adenosine (10γ to 20γ per c.c.) had been added, incubated at 37° C. showed much loss of activity after an hour and had lost all or nearly all its estimable activity after 1½ hours. Samples of sera to which 10γ of adenosine per c.c. were added lost all their activity after standing at room temperature for less than 72 hours. Fig. 12 shows the response to 1 c.c. doses of a sample of serum (six weeks old) to which 10γ of adenosine per c.c. had been added after incubation at 37° C. for 60 minutes (S_1), and 120 minutes (S_2). The injections indicated by arrows are of 10γ adenosine in 1 c.c. of saline and that at S_3 is to 10γ of adenosine freshly added to 1 c.c. of serum. The loss in activity on incubation can be clearly seen.

Part 3. The Brodie Effect.

The sharp fall of systemic blood pressure in the cat caused by the injection of serum and protein solutions was described by Brodie (1900), and according to this author is greatly diminished by atropinization or section of the vagi. There was considerable variation in sensitivity of our cats, some giving a sharp fall of systemic blood pressure after the injection of 1 c.c. of serum while others gave only a small transient fall with as much as 10 c.c. Division of the vagi or atropinization did not appear to affect the sensitivity. Generally speaking heterologous serum was more active than homologous serum. The latter was however quite active in producing the effect even when given to the very same cat which had originally provided the serum.

The initial fall of systemic blood pressure usually occurs between 5 and 30 seconds after the injection. The return to the original level occurs within the first two minutes or is delayed for 10 to 20 minutes or is occasionally terminated by death. During the fall the amplitude of the oscillations of mercury in the manometer usually decreases and may disappear altogether. With recovery there is sometimes a quickening of the heart beat and a temporary rise of systemic blood pressure. When this occurred in our experiments, usually the blood in the arterial cannula appeared to be imperfectly oxygenated. Subsequent injections of serum usually caused progressively smaller responses, and for comparison purposes sera were administered in alternate doses (see Fig. 15). For this reason also it was not possible to compare adequately in the same cat the effect of an injection of serum both before and after division of the vagus nerves.

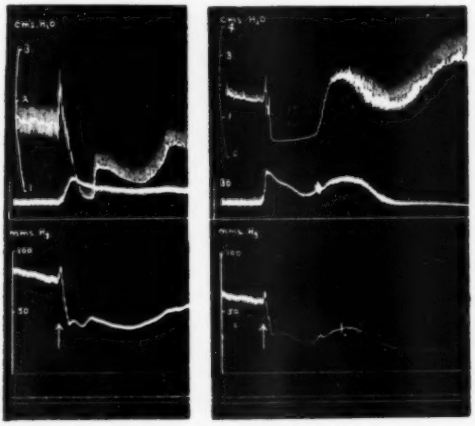

Fig. 13. Record of pressure in pulmonary vein (upper) pulmonary artery (middle) and carotid artery (lower) in two different cats with divided vagi under chloralose anaesthesia and artificial respiration. At the arrows 5 c.c. of cat serum were injected. Time in half-minutes.

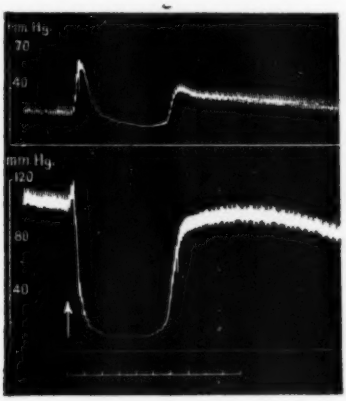

Fig. 14. Carotid and pulmonary blood pressure of a cat with vagi cut under chloralose anaesthesia and artificial respiration. At the arrow 5 c.c. of cat serum were injected. Time in half-minutes. Details in text.

The fall in systemic blood pressure is accompanied by a sharp rise in the pressure in the pulmonary artery and a fall in the pressure in the pulmonary vein. Fig. 13 shows the record of the pulmonary venous (upper) pulmonary artery (middle) and carotid artery (lower) pressures in two cats (weighing 2·6 and 2·9 kg. respectively) with vagi divided. After an initial volume effect the injection of 5 c.c. of cat serum from whole blood produced in both cats a rise in the pulmonary arterial and a sharp fall in the pulmonary venous pressures. The effect in the second cat was terminated by a cardiac failure, the pulsations in the carotid artery disappearing and the fall in pulmonary venous pressure giving place to a rise above the original level.

Fig. 14 shows how the initial rise in pressure in the pulmonary artery may occasionally be interrupted by a temporary cardiac failure. At the arrow 5 c.c. of cat's serum were injected into a cat weighing 2·9 kg. with vagi divided under artificial respiration and chloralose anaesthesia.

Intravenous injections of serum both homologous and heterologous were also given to decapitated and to decapitated and pithed animals. The fall in systemic blood pressure could regularly be produced in such cats. With the pithed animals, when the fall was profound and associated with anoxaemia of the blood in the arterial cannula recovery did not occur but on opening the thorax and applying cardiac massage they would recover.

An attempt was made to determine the direct action of serum on the isolated perfused cat's heart using Gunn's modification of Langendorff's method. We found, however, that injection of 2–5 c.c. of serum into the Ringer's solution just proximal to the aortic cannula had a dynamic effect, the heart increasing in rate and in the amplitude of its beats as recorded on a smoked drum by a lever attached by a thread to the apex. Usually this dynamic action which persisted for about five minutes was associated in the first 1–2 minutes with some coronary constriction. Both serum from whole blood (cat or human) and Hardyized human serum produced these effects.

Following the intravenous injection of serum in the cat Brodie described an increase in volume of the bowel and hind limb. We investigated the direct effect of serum on the splanchnic vessels by perfusion of the isolated mesentery. No evidence of a local vasodilator action was found, 2 c.c. doses of active human serum being usually without effect and 5 c.c. doses produced a transient vasoconstriction. Thus in one experiment the number of drops per half minute before the injection of 5 c.c. of human serum from whole blood was 54 and in each sucessive half minute following were 39, 28, 36, 43, 45, 48, 50 and 52.

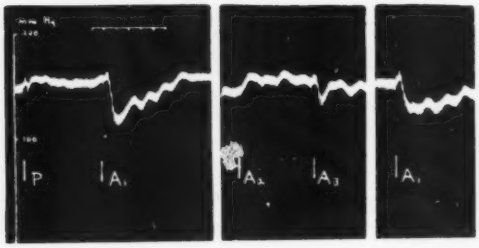

Fig. 15. Effect of injections of cat plasma and sera on the carotid blood pressure of a cat with divided vagi under choralose anaesthesia with artificial ventilation. The intervals between the panels were 7 and 3 minutes. Time in half-minutes.

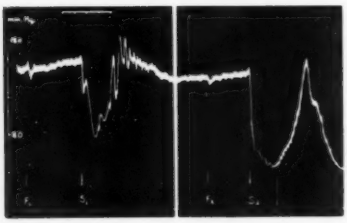

Fig. 16. Carotid blood pressure of a cat with vagi intact under chloralose anaesthesia and artificial respiration. Time in half-minutes; the interval between the panels, five minutes. Details in text.

Analysis. Brodie considered that the fall of systemic blood pressure following the intravenous injection of serum in the cat was due to reflex cardiac inhibition brought about through stimulation of afferent nerves in the lungs. After division of the vagi he found that the fall of blood pressure was much diminished and was then apparently due to a peripheral vasodilation in the limbs and viscera (except the kidney). The origin of such vasodilation he did not determine. We found no evidence of any direct vasodilator action on the mesenteric vessels so that Brodie's vasodilation may well have been central in origin or in those cats with intact vagi have arisen also reflexly from the lungs. Nevertheless in our experience the fall of systemic blood pressure occurred not only after vagal division but after decapitation and pithing and we concluded that the chief effect of the intravenous injection of serum was a direct one on the pulmonary and to a less degree on the coronary blood vessels. It should be pointed out however that a certain proportion of our cats suffered from pulmonary infection of possible virus origin which has been prevalent in this part of Australia for the last two years, but that there appeared to be no relationship between sensitivity to serum and the presence or absence of macroscopic pathology in the lungs. The animals whose blood pressure records have been used as illustrations had healthy lungs.

The constituent of serum responsible for the Brodie effect. The intravenous injection of homologous citrated plasma was without effect and heterologous plasma though frequently producing a small response was usually much less active than the corresponding serum prepared from whole blood to which citrate was added. Homologous serum prepared by adding calcium chloride to citrated platelet-rich plasma was nearly always more active than serum similarly prepared from centrifuged citrated plasma but this difference could not always be demonstrated with heterologous sera.

Fig. 15 shows the effect of the intravenous injection of cat sera and plasma into a cat weighing 3·0 kg. with divided vagi, under chloralose anaesthesia and artificial respiration. Two c.c. doses were injected of centrifuged citrated cat's plasma at P, serum from whole blood to which citrate was added at A_1, serum from centrifuged plasma at A_2 and serum from gravity plasma at A_3. The whole of the activity of homologous serum appears therefore to be related to the clotting process and the difference in activity between serum from platelet-rich plasma and serum from platelet-free plasma suggests that the platelets are in some way responsible. We found

that unboiled platelet extracts possessed activity in producing the Brodie effect which was much greater than could be accounted for by the plasma element which might still be present. Boiled platelet extracts were nearly always without activity in doses equivalent to 20 c.c. serum.

This latter observation suggests that the effect of an intravenous injection of serum cannot be due to the heat stable vasoconstrictor substance already described and this view is further supported by the following results:

1. There was often complete absence of parallelism between the effects of different sera on the ox carotid and guinea-pig jejunum on the one hand and on the cat's blood pressure on the other.

2. Hardyized human serum, without muscle-stimulating activity was quite active in producing the Brodie effect and was only somewhat less active in this respect than the serum from which it was prepared.

3. Filtrates of serum (homologous and heterologous), prepared after boiling, and ultrafiltrates, while active on the ox carotid or guinea-pig jejunum, were without activity on the cat's blood pressure.

Fig. 16 shows the record of the carotid blood pressure of a cat weighing 2·9 kg. with intact vagi under chloralose anaesthesia and artificial respiration. At S_1, 4 c.c. of human serum and at S_2, 4 c.c. of cat's serum were injected intravenously. At F_1 and F_2 corresponding volumes (10 c.c.) of the filtrate from the same sera after dilution, boiling and filtering were also injected. When tested on the ox carotid, these filtrates were as active as the sera to which they corresponded.

4. Dialysed serum which had no action on the ox carotid or guinea-pig jejunum gave a good response when injected into a cat (Fig. 17).

The effect of the intravenous injection of serum appears to be a specific one in the cat (Brodie, 1900). We could not obtain the effect in dogs or rabbits using doses up to 20 c.c.

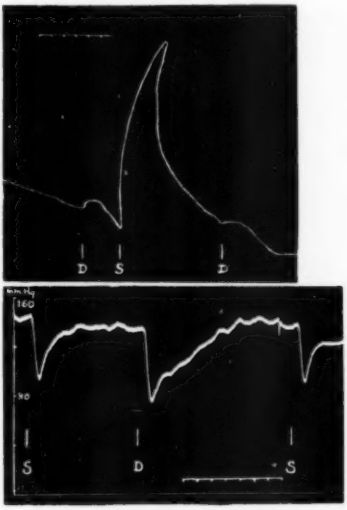

Fig. 17. Upper tracing shows the response of the ox carotid to 2 c.c. of human serum at S and to a corresponding volume after dialysis at D. Time in minutes. Lower tracing shows the effects on the carotid blood pressure of a cat with vagi cut under chloralose anaesthesia and artificial respiration to 2 c.c. doses of the same sera. Time in half-minutes.

DISCUSSION.

Our results confirm earlier observations that muscle stimulating and vasoconstrictor substances are liberated into serum during the process of clotting. Of the elements which take part in the clotting process those present in the plasma are clearly not responsible, for platelet-free plasma yields an inactive serum while washed platelets yield an extract which produces the effects of active serum on the various test objects and is as active quantitatively as is serum prepared from the original platelet containing plasma. Indeed, the actual clotting process is unnecessary for the liberation of the pharmacologically active substances; it is platelet breakdown which is essential and plasma rich in platelets may be made fully active by mechanical shaking.

Although the work of Daly and Thorpe (1933) suggests that part of the vasoconstrictor activity of defibrinated blood or sera may be attributable to particulate matter removeable by cambric filters, this cannot account for the action of sera on isolated strips of carotid or intestine. Moreover the active substances can be obtained in ultrafiltrates and are absent from dialysed serum.

Of the various preparations used, the ox carotid, the rabbit's ear and the jejunum of the guinea-pig gave consistent results. In all of these preparations platelet extracts and serum prepared from blood or platelet-rich plasma produced contraction or constriction whereas plasma, Hardyized serum and serum prepared from platelet-free plasma produced at best only trivial responses. In the rabbit's ear viscosity contributed to the effect of injected solutions. Results with the uterus of the rat while usually agreeing with those obtained with other preparations showed some inconsistencies, e.g. sometimes inactive Hardyized serum produced a good response. Such responses were probably due to foreign protein and recall the results of Stewart and Zucker

(1913) who found that whereas the ox carotid responded to serum only, the intestine or uterus of the rabbit often responded to both plasma and serum. These workers concluded that the substance liberated in the clotting process was specifically vasoconstrictor and contributed to haemostasis.

Bornstein (1926) stated that vasoconstrictors in defibrinated blood disappear in about 24 hours. Anrep and Häusler (1928) could not confirm this finding. Bayliss and Ogden (1933) using the pump-oxygenator kidney preparation described substances in defibrinated blood which produced "temporary" and "permanent" effects on the kidney vessels, the former persisting indefinitely the latter disappearing after 48–72 hours. Our experience has been that serum at room temperature retains its activity for at least 3 months. On the other hand platelet extracts and ultrafiltrates lose their activity very rapidly, even at 0–2° C.

The active substances must possess molecules small enough to pass through celloidin or cellophane membranes, a fact previously described by Janeway, Richardson and Park (1918). Several other authors have shown that they are resistant to boiling (Hashimoto (1928), Janeway, Richardson and Park (1918)). Stewart and Harvey (1912) described vasodilator and vasoconstrictor substances in dog's serum, the former protein in nature and destroyed by boiling, the latter a substance which resisted boiling.

The failure of the ox carotid to respond to the ether and alcoholic extracts of dried serum agrees with the findings of Stewart and Zucker (1913) and Kaufmann (1913). From the records of workers who obtained active alcoholic or ethereal extracts (Zucker and Stewart (1913), Janeway, Richardson and Park (1918)) it would appear that the extracts were quantitatively much less active than the corresponding sera. The inactivity of the reconstituted protein fraction of serum, prepared by Hardy's method, is in agreement with the heat stable and dialysable properties of the muscle-stimulating substances.

The large quantities of adenylic acid which Zipf (1931) obtained from defibrinated blood were probably derived from leucocytes broken down in the preparation of protein free filtrates from such blood. Our failure to demonstrate any detectable adenyl compounds is due to the presence of an enzyme in serum which brings about their breakdown. It is perhaps significant in this respect that Freund's (1920) vasodilator substance in defibrinated blood disappeared within half-an-hour.

The fall of systemic blood pressure in the cat following an intravenous injection of serum occurs also in the decapitated and pithed animal and is produced by a direct constriction of pulmonary arteries. Vasoconstrictor substances in serum, it is true, may cause pulmonary and coronary vasoconstriction but the Brodie effect is, for reasons already stated, not related to these substances. Nevertheless all the activity of homologous serum is related in some way to the clotting process and the difference in activity between serum prepared from platelet-free and platelet-rich plasma suggests that the platelets may play a part. However the substance derived from such platelets is destroyed by heating to 100° C. and is probably as Brodie considered, a protein. This view is further supported by the activity of Hardyized serum and the inactivity of serum filtrates. Heterologous plasma is not necessarily devoid of activity, indeed it seems as though a variety of foreign proteins may produce the Brodie effect, e.g. egg albumin (Brodie, 1900).

So far as human transfusions are concerned, conclusions as to the importance or otherwise of the pharmacologically active substances in serum must ultimately be based on clinical experience. Inferences drawn from experiments such as those of Buttle *et al.* (1940) in which serum was given to cats in the treatment of experimental haemorrhage are probably misleading. The results described above show that the cat (but not the dog or rabbit) reacts towards its own serum in a special manner. So pronounced indeed is this reaction with even small doses of serum that if there were any evidence that man behaved in an analogous fashion the intravenous use of human serum would certainly be out of the question.

When one considers reports on the inferiority of serum relative to plasma based on results of transfusions in man one finds that either insufficient cases were observed (Black, 1940) or that concentrated serum was used (Aldrich *et al*, 1938; Black, 1940; Brown and Mollison, 1940) or the possibility of pyrogens could not be excluded. It is perhaps significant that the reactions described with serum have been mainly febrile and did not differ in type from the reactions sometimes occurring with plasma or whole blood (fresh or stored). It is difficult to see how the muscle-stimulating substances could be responsible for such reactions. In point of fact numerous reports (Wright *et al.*, 1938; Clegg and Dible, 1940; Hill *et al.*, 1940 and Bick and Drevermann, 1940) indicate that human serum (properly prepared from either whole blood or from centrifuged plasma), is quite safe.

Our results do, however, indicate that relatively simple methods are available for the preparation of serum which contains pharmacologically active substances in minimal amounts. Such are the methods at present in common use, in which serum is prepared from centrifuged or "cream-separated" plasma. The latter although more active than the former has relatively little activity when compared with serum from whole blood or gravity plasma. Reconstituted Hardyized serum prepared from whole blood is quite devoid of muscle stimulating or vasoconstrictor activity.

SUMMARY.

A vasoconstrictor and smooth muscle-stimulating substance (or substances) is liberated into serum in the process of blood clotting.

This substance exists already preformed in the platelets and its liberation is incidental to the clotting process. Accordingly active platelet extracts can be prepared and platelet-rich plasma may be activated by mechanical shaking.

Serum retains its vasoconstrictor activity for at least three months. Ultrafiltrates and platelet extracts lose their activity much more rapidly.

The vasoconstrictor substance is not extractable with ethyl alcohol, ether or methyl alcohol. It resists boiling, will dialyse through cellophane and may be obtained in an ultrafiltrate.

Serum prepared from plasma which has been centrifuged to free it from nearly all its platelets has little or no vasoconstrictor or muscle-stimulating activity. Serum prepared from "cream-separated" plasma has more activity than that prepared from centrifuged plasma but is much less active than that prepared from whole blood. Hardyized serum is inactive.

Active adenyl compounds are not present in human serum (more than 12 hours old) because such serum regularly contains an enzyme which inactivates adenosine.

The intravenous injection of homologous or heterologous serum made from whole blood produces in the cat (but not in the dog or rabbit) a fall in systemic blood pressure which can still be obtained after division of the vagi, atropinization, decapitation or pithing, and is due mainly to direct pulmonary vasoconstriction. The vasoconstrictor substances described above are not responsible for this effect but it can be caused by a number of protein substances as well as a heat-labile, non-dialysable substance which is liberated into serum with the process of clotting and is derived from the platelets.

The importance or otherwise of these pharmacological properties of serum so far as human transfusions are concerned, is discussed.

Acknowledgments. We are indebted to Dr. C. H. Kellaway for his interest and advice, to Mr. H. F. Holden for some of the samples of dried serum, to Dr. Hilda Gardner who performed the platelet counts and to Miss Beryl Splatt for the ionic determinations.

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THE ESTIMATION OF BARBITURIC ACID DERIVATIVES

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The methods employed for the estimation of barbiturates are modifications of the cobalt colour reaction (Koppanyi, 1934). From personal experience I am in agreement with the statement of Kozella, Nelson and Tatum (1939) that this method is only semi-quantitative. Krause and Riley (1941) while improving the development of the colour with cobalt salts have noted, in agreement with Riley *et al.* (1940), that there are other bodies present in urine which develop a colour equivalent to 20 mg. of amytal per 100 ml. of urine. Therefore, a quantitative method has been devised in which the barbituric acid derivative is precipitated as the silver salt and the silver estimated volumetrically. Difficulties, due mainly to the presence of chlorides and other bodies, were encountered when this method was applied to body fluids, but they have been eliminated by suitable methods.

The principle of the method is that the solution containing the barbiturate is rendered alkaline, giving rise to the salt of the acid, which is insoluble in ether and chloroform, and this enables a separation from ether-soluble substances to be made. The solution is then rendered acid and the free barbituric acid is now extracted with an ether-chloroform mixture, the latter is removed by evaporation, the barbiturate is taken up in alkali water and precipitated as the silver salt, which is determined by ammonium thiocyanate.

The solubility of silver barbiturate in water is dependent on the hydrogen ion concentration. It is completely precipitated between pH 7·5 and pH 5·5, the solubility increases slowly to pH 4·4 and then increases rapidly.

EXPERIMENTAL.

The volume of the liquid should be between 5 and 20 ml. and contain from 1 to 10 mg. of barbiturate. The liquid is first roughly neutralized, 1 ml. of N NaOH is then added and it is allowed to stand for 5 minutes. It is then shaken in a separating funnel with 4 volumes of a mixture containing 75 p.c. ether and 25 p.c. chloroform. To the separated watery layer are added 2 ml. of N H_2SO_4 and it is allowed to stand for 5 minutes. It is now extracted twice with 4 volumes of the ether-chloroform mixture. Care must be exercised in shaking with the extraction mixture to avoid emulsification. This is especially so in the case of blood, when a gentle rocking to and fro is advised. The combined ether-chloroform layers are evaporated or distilled on a water-bath. The dish must be removed from the bath as soon as evaporation is complete. 2 ml. of water and 0·2 ml. of N NaOH are added to the dish which is gently rotated to dissolve the barbiturate, the liquid is poured into a pointed centrifuge tube of about 5 ml. capacity and centrifuged. The supernatant fluid is transferred to another centrifuge tube. The evaporating basin is rinsed with 1 ml. of water, which is poured into the first centrifuge tube, centrifuged, and the liquid transferred to the second centrifuge tube. 0·2 ml. of an alcoholic solution of phenolphthalein is added and two drops of N acetic acid. 0·1 N acetic acid is now carefully added dropwise until the solution is just colourless. 0·5 ml. of 5 p.c. silver nitrate is added and after 2 minutes the tube is centrifuged. The liquid is poured off and the tube is washed 3 times with ice cold water, if possible without disturbing the precipitate. If the wash water should disturb the precipitate it will be necessary to recentrifuge. After the last wash water has been poured off, 3 ml. of 1 p.c. HNO_3 are added to the tube and the precipitate agitated till it has almost completely dissolved. The liquid is transferred to a flask and the tube rinsed with a little water which is also poured into the flask. Two to three drops of iron indicator (prepared by oxidizing 100 ml. of a saturated solution of ferrous sulphate with 50 ml. of concentrated nitric acid, boiling to expel the nitric fumes) are added and the solution is titrated with N/50 NH_4CNS, freshly prepared from a stock solution of N/10 NH_4CNS, until a permanent brownish tinge is apparent. Slight impurities

[1] This Research has been aided by technical assistance provided by the National Health and Medical Research Council, and expenses were defrayed by a grant from the Fletcher Bequest.

have been encountered in the ether and chloroform which make the carrying out of a blank advisable.

1 ml. of N/50 NH_4CNS is equivalent to the molecular weight of the barbiturate acid derivative divided by 100 and expressed as mg.

The following derivatives have been added to urine in varying amounts and have been recovered quantitatively: Veronal (Mol. wt. 184), Luminal (232), Dial (208), Nembutal (226), Amytal (226), Pentothal (265) and Evipan (256). The recovery of Seconal (273) was not so satisfactory, averaging about 70 p.c. In a series of experiments 2 mg. of Luminal were added to 10 ml. of urine and the recovery was 2 mg. $\pm$ 0·22. To obtain the molecular weight of the sodium salt add 22 to the figures in the brackets.

Blanks carried out on 20 urines (man) gave a very constant value equivalent to 3 mg. of barbiturate per 100 ml. of urine, and it would be permissible to subtract this amount from all determinations of barbiturates in urine. (In clinical cases interference by other medicaments must be considered.)

Recoveries of added barbiturates from blood were not quite so satisfactory. This is very often the case in micro estimations and is not surprising because the opportunities for adsorption and destruction are greater. However the recovery of the above mentioned drugs varied between 80 and 95 p.c.

Attention may be drawn to two articles published recently in which methods are described for the extraction of barbiturates from blood and tissues which may be used in conjunction with the method described above: Kozella *et al.* (1939) and Levvy (1940).

SUMMARY.

A method is described for the micro-estimation of barbituric acid derivatives. It is applicable to body fluids.

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TISSUE INJURY BY TRYPSIN

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(Accepted for publication 2nd February, 1942.)

INTRODUCTION.

Rocha e Silva (1940) has shown that histamine is liberated from the isolated perfused lung of the guinea-pig following the intra-arterial injection of trypsin. He has also shown that the isolated jejunum of the guinea-pig responds to trypsin by contraction after a delay of some seconds and following this relaxation is gradual. Feldberg, Holden and Kellaway (1938) were able to demonstrate that a "slow-reacting muscle stimulant substance" was formed by the action of cobra venom upon egg-yolk and Holden (1938) showed that trypsin acted similarly. Since the liver of the rabbit is almost free of histamine it is a suitable organ for investigating the liberation of a muscle-stimulant substance other than histamine such as the "slow-reacting muscle-stimulant substance" described by Feldberg and Kellaway (1938), and since it has been shown (Kellaway and Trethewie, 1940a) that cobra venom causes the liberation of adenyl compounds and an inactivating enzyme from the rabbit's liver it was thought worth while to determine if trypsin, also an external glandular secretion, could produce these effects.

METHODS.

Perfusion experiments. After either intravenous chloralose (11 c.c. of 1 p.c. solution per kg.) or ether anaesthesia the livers of rabbits were excised and perfused via the portal vein at a rate of 2 c.c. per minute with oxygenated Tyrode solution as described for the dog by Feldberg and Kellaway (1937). Injections of trypsin (Difco 1: 250) (40 mg. in 1 c.c. Tyrode) were made into the intraportal cannula directly and the flow interrupted for 2 minutes. Unless otherwise stated samples were collected over a boiling water bath 15 minutes before and 0–3, 3–10, 10–30 and 30–60 minutes after injection.

Extracts of organs. Extracts of organs were made by grinding up the organ thoroughly with silica in Tyrode solution and also immediately boiling when adenyl compounds were estimated. Control extract refers to extract made from portions of the perfused liver other than the lobe used in the experiment.

Biological assay. Tests of trypsin, unboiled extracts of organs and perfusate and histamine hydrochloride were made upon the isolated jejunum of the guinea-pig suspended in a 7 c.c. bath of oxygenated Tyrode solution. Extracts and perfusate after boiling were tested on the guinea-pig heart preparation for adenyl compounds and estimated as adenosine as described by Drury, Lutwak-Mann and Solandt (1938).

RESULTS.

The Liberation of a Slow-reacting Muscle-stimulant Substance by Trypsin from the Isolated Perfused Liver.

(a) *Response of the isolated jejunum of the guinea-pig to trypsin.* It has been shown by Rocha e Silva (1940) that trypsin causes contraction of the guinea-pig jejunum which occurs after some seconds or 1–2 minutes and following this relaxation is gradual. This finding is confirmed. With Difco trypsin in a dose of 0·5–10 mg. contraction of the gut occurs after a latent period of 20–50 seconds. The contraction is slower in development than that due to histamine and usually increases when the Tyrode solution in the bath is changed whether it be 30 or 60 seconds after injection. Following the response to trypsin the extent of contractions to histamine is depressed, but if 4–6 minutes after the injection of trypsin histamine is tested again there is frequently an increased response with delay in relaxation on washing out. Responses to later doses of trypsin are diminished. These findings are illustrated in Fig. 1. In the top left hand panel the response to 0·5 mg. trypsin occurred after an interval of 30 seconds (T). Succeeding

[1] This work was aided by a grant from the Australian National Health and Medical Research Council.

responses to histamine (0·1γ) were depressed and after the third injection of histamine relaxation was gradual following an increased response. In the right hand panel a "slow-reacting" response is seen following washing out the bath (W), six minutes after testing 10 mg. trypsin at TI, which was left in contact with the gut for 1 minute. In the lower panel of Fig. 1 a similar response following the injection of 0·1γ histamine 5 minutes after the injection of 5 mg. trypsin (TII) (in contact 1 minute) is seen (at H). Thereafter the sensitivity to histamine was depressed and no response was observed following a second injection of 5 mg. trypsin 32 minutes after the first (TII).

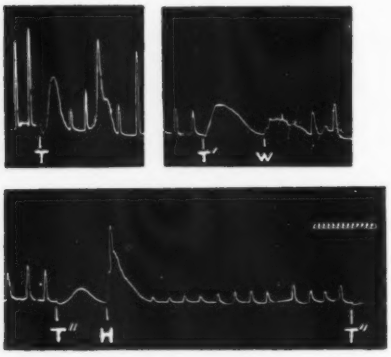

Fig. 1. Responses of the isolated jejunum from three guinea-pigs to trypsin. The unlettered contractions are to 0·1γ histamine. Time in half-minutes. Details in text.

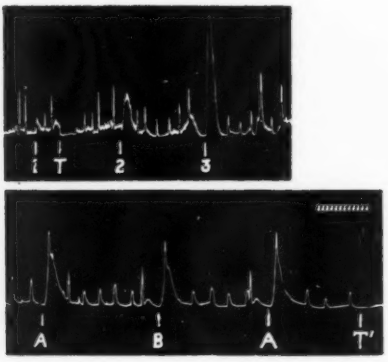

Fig. 2. Responses of the isolated jejunum from two guinea-pigs. Upper panel: tests on perfusate. Lower panel: tests on organ extracts. Unlettered contractions to 0·1γ histamine. Time in half-minutes. Details in text.

(b) *Perfusate from the liver.* Following the injection of trypsin intraportally in the isolated perfused liver of the rabbit there is an output into the perfusate of a substance with an action on the guinea-pig jejunum similar to that described by Feldberg and Kellaway (1938) and Feldberg, Holden and Kellaway (1938) as formed by the action of cobra venom on the isolated organ and upon egg-yolk. This substance was usually in greater concentration in the sample obtained 10–30 minutes or 30–60 minutes after injection, and the output was more readily detected when ether anaesthesia was employed than with chloralose. The slow-reacting response of the guinea-pig jejunum is not due to contamination by trypsin because the responses to samples of perfusate were obtained either on a gut desensitized to trypsin or on one which was insensitive to trypsin in the dose present in the testing sample if all of the trypsin had passed into the perfusate. Further, the samples containing the most trypsin did not have the greatest effect upon the isolated jejunum. These findings are shown in Fig. 2 where a portion of liver weighing 10·3 gm. from a rabbit (2·5 kg.) was perfused at 1·6 c.c. per minute and 40 mg. trypsin were injected. 1 c.c. of the sample obtained before injection (1) had negligible effect on the gut. At *2* and *3* 1 c.c. of the samples obtained 30–60 and 10–30 minutes respectively after injection produced contractions of the gut with latencies of seven seconds, and on changing the Tyrode solution after 30 seconds relaxation was only gradual. 1 mg. trypsin, the maximum amount which could be present in 1 c.c. of either sample 2 or 3 were all the trypsin to have passed into the perfusate, produced only some inhibition of the rhythm of the gut at (T). Following the slow-reacting response "after-changes" in the excitability of the gut to histamine are seen as has been described by Feldberg and Kellaway (1938).

(c) *Organ extracts.* Simple unboiled saline extracts after injection when compared to similar extracts of uninjected liver showed a slight diminution in activity of slow-reacting substance (S.R.S.) but the difference was hardly significant. This is shown in the lower panel of Fig. 2 in an experiment where a piece of liver weighing 12·1 gm. was injected intraportally with 40 mg. trypsin. At A the response to extract of 1·25 mg. of control liver is compared with 1·25 mg. of liver extract obtained after injection and perfusion for 1 hour (at B). The extracts were left in contact with the gut for 30 seconds and the latencies were 17, 20 and 21 seconds respectively. Typical "after-changes" in excitability are seen in the responses to histamine. It is seen that a slight decrease in activity of the liver is detected after injection so that

this evidence points to the fact that the slow-reacting substance is liberated rather than formed by the action of trypsin on the liver in perfusion experiments. 5γ of trypsin, the amount possibly present in sample B tested at T_1 was without effect on the gut. The addition of trypsin in this dose to liver extract without incubating was also without effect on the size of the response to the gut. It might be argued that the method of extraction, simple grinding, is not complete so that such a comparison of extracts is not allowable. However it has previously been shown (Kellaway and Trethewie, 1940b) that S.R.S. is more readily extracted by this method from tissues injured in anaphylaxis than from control tissue though when the S.R.S. was completely extracted by vacuum desiccation the content of control and injured tissue was seen to be the same. Therefore the greater content of the normal tissue here shown may be significant. Further the content of the normal tissue obtained by thorough grinding is quite high as shown by the responses to such a small amount of tissue as 1·25 mg. in Fig. 2. Finally this result was consistently obtained. Holden (1938) has shown that following the treatment of egg-yolk with trypsin a slow-reacting muscle-stimulant substance can be extracted.

The Liberation of Adenylic Acid from the Isolated Perfused Liver.

The isolated liver removed from the rabbit under chloralose anaesthesia can be perfused via the portal vein with oxygenated Tyrode solution for one and a quarter hours without the appearance of cardio-inhibitory substance in the perfusate as detected in the guinea-pig heart preparation though when ether is used traces equivalent to 1 up to 2·5γ adenosine per c.c. may be detected.

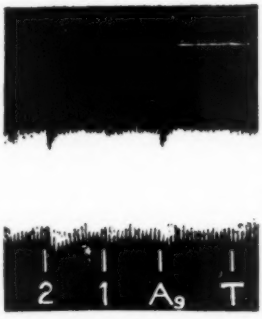

Fig. 3. Responses of the right auricle of the atropinized guinea-pig to perfusate. At A_9 response to 9γ adenosine. Time in half-minutes. Details in text.

Following the injection of trypsin into the portal vein of the perfused rabbit's liver adenyl compounds are liberated into the perfusate. The first sample, obtained 0–3 minutes after injection, is usually free of cardio-depressant activity, but thereafter increasing amounts are present. The absence of adenyl compounds from the first and frequently from the second sample may be due to some delay in action of the trypsin, but can equally well be due to destruction of liberated adenyl compound for it can be shown that when trypsin is mixed with adenosine in a concentration of 5 mg. trypsin per c.c. there is an immediate slight diminution of the cardio-depressant activity of the mixture followed by increasing loss on incubation. Following the appearance of cardio-depressant activity in the perfusate three or ten minutes after the injection of 40 mg. of trypsin the output is well maintained, varying from 5–14γ adenosine per minute during the second half hour after injection.

The findings in an experiment where a rabbit weighing 2 kg. was anaesthetized with chloralose and a portion of perfused liver weighing 13·3 gm. was injected intraportally with 40 mg. trypsin are shown in Fig. 3. It is seen that 1 c.c. of the sample obtained before injection (*1*), and 8 mg. of trypsin (boiled) (T) are without cardio-depressant activity when tested on the atropinized guinea-pig heart preparation. 1 c.c. of the sample obtained 30–60 minutes after injection and boiled (*2*) is seen to have cardio-depressant activity equal to that of 9γ adenosine (A_9). The maximum amount of trypsin which this sample could have contained were it all to have passed into the perfusate is 0·5 mg. per c.c. The cardio-depressant activity therefore cannot be due to injected trypsin. The liver after perfusion weighed 13·7 gm., a slight increase (3 p.c.), and contained cardio-depressant activity equivalent to 0·7 mg. adenosine per gm. A control sample of uninjected liver contained the equivalent of 1·05 mg. adenosine per gm.

TABLE 1.

The loss of adenyl compound from the isolated perfused liver and output in the perfusate following the injection of trypsin.

Number of experiment.	Content of control liver extract as mg. adenosine per gm.	P.c. loss adenyl compound from liver after injection.	Output in perfusate as γ adenosine.	Output as γ per minute.
1	1·05	30	496	8·3
2	1·0	28	398	6·6
3	1·1	42	340	5·8
4	0·8	39	512	8·5
5	1·3	51	681	11·3
6	1·3	54	*318	10·6

* Perfused for half an hour only.

The findings in this and two similar experiments are shown in Table 1 (1, 2 and 3 respectively). Now when the animal is anaesthetized with ether (experiment 4, 5 and 6 in Table 1) the perfusate obtained from the liver before injection of trypsin contains a trace of cardio-depressant activity equivalent up to 2·5γ adenosine per c.c. It has been shown previously (Trethewie, 1941) that chloroform causes the liberation of adenyl compounds from the perfused and intact liver of the dog and it is possible that the small output following ether anaesthesia is due to injury by the volatile anaesthetic. In addition it is found that the output of adenyl compounds is greater following the injection of trypsin after ether anaesthesia than after chloralose, and the loss in adenyl compound from the organ is also greater (Table 1, experiment 4, 5 and 6). The average output of adenyl compound was 6·7γ per minute when chloralose was used and 10·1γ per minute with ether. The corresponding averages in adenyl compound content of the liver were 30 p.c. and 48 p.c. respectively. In this connection it is noteworthy that Dale (1919) showed that histamine shock is more readily produced in cats under ether anaesthesia, than when they are unanaesthetized.

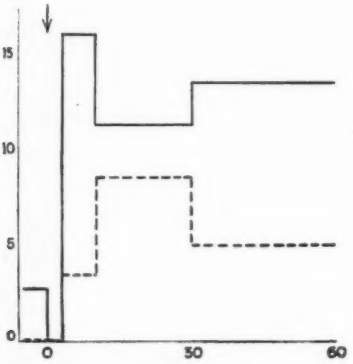

Fig. 4. Output of adenyl compound estimated as adenosine from two livers perfused with Tyrode solution. Continuous line: ether anaesthesia, interrupted line: chloralose anaesthesia. At the arrow 40 mg. trypsin injected intraportally in each. Ordinates: output of adenosine in γ per minute. Abscissae: time in minutes. Further details in text.

In Fig. 4 are shown graphically the outputs of adenyl compound as γ adenosine per minute following the injection (at the arrow) of 40 mg. trypsin intraportally into pieces of liver from two rabbits when ether anaesthesia was used (continuous line) and when chloralose was used (interrupted line). It is to be noted that the small initial output in the case of ether anaesthesia fell to zero immediately following injection, and this is to be attributed to inactivation of adenyl compound by the high concentration of trypsin present in the first sample obtained after injection.

The Liberation of Inactiviting Enzyme from the Isolated Perfused Liver.

It is found that the output of adenylic acid in the perfusate is much less than the loss from the organ as determined by difference of organ extracts, e.g. in the experiment of line 1 in Table 1 the loss from the organ in adenyl compound (equivalent to 4·7 mg. adenosine) is far in excess of that detected in the perfusate (0·5 mg.). This is to be explained by the presence of inactivating enzyme in the liver which is liberated into the perfusate as occurs with cobra venom (Kellaway and Trethewie, 1940a), and which may also act in the liver cells of the damaged organ. This was investigated as follows. A piece of liver weighing 9·5 gm. was perfused with Tyrode solution and the perfusate was allowed to fall on a knife edge which divided the outflow into two parts, one of which was collected at room temperature, and the other over a boiling water bath. After thirty minutes when 11·5 and 13·8 c.c. respectively of perfusate were collected 40 mg. of trypsin were injected intraportally and during the succeeding half hour 13·7 and 5 c.c. of perfusate respectively were collected. The perfusate collected over the boiling water bath after injection contained cardio-depressant activity equivalent to 17γ adenosine per c.c. whereas that collected before contained only a trace. To the unboiled perfusate, which had been allowed to stand at room temperature, adenosine was added in a concentration of 1: 55,000. In addition trypsin (14 mg. to 10 c.c. perfusate) was added to the sample obtained before injection in the same concentration as it would be present in the sample obtained after injection were all of the trypsin to have passed into the perfusate. A portion of each mixture was immediately boiled, and the remainder incubated at 38° C. for 30 minutes and then boiled. The added adenosine

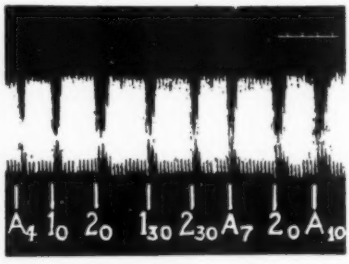

Fig. 5. Responses of the right auricle of the atropinized guinea-pig to perfusate. At A_4, A_7, A_{10} responses to 4, 7 and 10γ adenosine. Time in half-minutes. Details in text.

should account for 9γ in 0·5 c.c. of the perfusate. It is seen in Fig. 5 that the perfusate collected before injection of trypsin (1_0) contains the equivalent of 7γ adenosine in 0·5 c.c. before incubation and this apparent fall is due to the presence of trypsin, but this is not further reduced during 30 minutes incubation (1_{30}). When small amounts of trypsin, up to 1 mg. per c.c. are mixed with adenosine 1: 100,000 there is no immediate reduction of activity, but with doses of trypsin up to 5 mg. per c.c. there is a slight immediate reduction of cardio-depressant activity when tested on the guinea-pig heart preparation of 15–30 p.c. With doses of 5 mg. trypsin per c.c. there is a further reduction in activity of the mixture on incubation, e.g. in one experiment there was an immediate reduction of 30 p.c. on mixing, which increased to 60 p.c. after fifteen minutes incubation at 38° C. and no cardio-depressant activity could be detected after 30 minutes in a mixture containing adenosine in a concentration of 1: 50,000 originally. It is also seen in Fig. 2 that the perfusate collected after injection of trypsin contained activity equivalent to 10γ adenosine in 0·5 c.c. (2_0). This increase on the added adenosine in spite of the presence of trypsin is due to the fact that not all the adenyl compound already present in the perfusate was destroyed while the sample was left to stand at room temperature. The original content was very high, equivalent to 17γ adenosine per c.c. Nevertheless a considerable reduction in activity is observed after incubation for 30 minutes from 10γ in 0·5 c.c. (2_0) to 6γ in 0·5 c.c. (2_{30}). Thus perfusate collected following the injection of trypsin was capable of inactivating 0·8 mg. of adenosine per 100 c.c. in 30 minutes at 38° C., whereas perfusate collected before the injection of trypsin caused no progressive diminution of cardio-depressant activity following incubation even when mixed with trypsin.

DISCUSSION.

Tissue injurious agents have been shown to cause the liberation of numerous pharmacologically active substances and though such a powerful agent as snake venom appears to liberate most of the substances investigated, histamine, "slow-reacting substance", lysocithin, adenyl compounds and inactivating enzyme (Feldberg and Kellaway, 1937a, 1938; Feldberg, Holden and Kellaway, 1938; Trethewie, 1939; Kellaway and Trethewie, 1940a), some types of injury are not so general as for example the gaseous anaesthetics which do not liberate S.R.S. (Kellaway and Trethewie (1939)) and the Cl. Welchii group of toxins some of which do not liberate histamine (Kellaway and Trethewie (1941a, b)). It has seemed worth while to investigate each agent as fully as possible so that in time it might be possible to ascribe the liberation of certain of the pharmacologically active substances to specific attributes of the injurious agent. This has been partly attained with the Cl. Welchii group of toxins (Glenny *et alia*, 1933; Kellaway, Trethewie and Turner, 1940; Kellaway and Trethewie, 1941a, b) and the ability to liberate histamine and "slow-reacting substance" by snake-venom has been paralleled by haemolytic activity while the neurotoxic action is evidently not so related (Trethewie, 1939). Feldberg and Kellaway (1938) earlier produced evidence that injury by snake venom can be explained at least in part by the formation of lysocithin which is haemolytic.

We have been able to show that trypsin is able to liberate a "slow-reacting substance" from the liver, and also adenyl compounds and inactivating enzyme. Rocha e Silva (1940) has previously shown that trypsin causes the liberation of histamine from the lung, and this has been confirmed and extended by Ramirez de Arellano, Lawton and Dragstedt (1940). The attempt to relate the action of the anaphylactic antigen solely to histamine or "slow-reacting substance" (Kellaway and Trethewie, 1940b) was unsuccessful. It appears almost certain that both are involved, whereas no evidence was obtained that the liberation of adenyl compounds plays a part in the liver in anaphylaxis in the guinea-pig (Kellaway and Trethewie, 1940c). However, trypsin does cause the liberation of adenyl compounds and so this factor is added to the symptoms produced from injury by trypsin. Rocha e Silva (1941) was unable to produce any cross desensitization with trypsin for the anaphylactic antigen or *vice versa*. He concludes that possibly the site of action of these injurious agents is different.

Trypsin is a proteolytic substance, and it would appear that it possesses the ability of so altering the permeability of the cell membrane and inner-cell structure

that a slow-reacting muscle stimulant substance, adenylic acid and an inactivating enzyme are liberated from the cell substance into the vessels as also occurs with histamine. Now all of these substances are present in the cell in the pre-formed state where they are not able to produce vascular, cardiac and smooth-muscle effects possibly because they are not in outside contact with these other situations. It may only be that trypsin is required to render the protecting membranes permeable, when they will be liberated since a pressure gradient exists, or that trypsin also acts in the cell on some inactive precursor in addition though information of the latter is lacking. It is noteworthy that we regard cell membranes and cell substance as made up of definite patterns of lipo-protein nature and that we have evidence of a proteoclastic enzyme (trypsin) which will allow the liberation of contained substances. Snake venom contains a powerful lipoclastic enzyme which will split off oleic acid from lecithin, a constituent of cell membranes, with the formation of lysocithin and Feldberg and Kellaway (1938) have suggested that this process, remembering also that lysocithin is powerfully haemolytic, may be the mechanism of liberation of substances from the cell by venoms.

Finally, in view of the similarity of the substances liberated by trypsin and by snake venom it is perhaps not surprising that few conditions produce so rapid and so severe a state of "shock" as severe snake bite and acute pancreatic necrosis, where pancreatic enzymes are liberated and the effects of digestion are observed in the abdomen and sometimes in distant parts of the body.

SUMMARY.

Trypsin causes the liberation of adenyl compound, an enzyme which will inactivate it, and a slow-reacting muscle stimulant substance from the isolated perfused rabbit's liver. The output of these substances is greater when ether anaesthesia has been employed than when chloralose is used. The significance of these findings is discussed.

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THE STEROL FRACTION OF AUSTRALIAN MARINE MOLLUSCA

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The sterol constituents of bivalves have been investigated by several workers. Cholesterol was reported to occur in *Mytilus edulis* (Daniel and Doran, 1926), and in a fresh water bivalve (de Waele, 1931). Bergmann (1934a) isolated from oysters and other mollusca an isomer of stigmasterol (ostreasterol), but did not report the presence of cholesterol. Later, the same worker (1937) occasionally detected small amounts of stigmasterol in oysters. Gillam and Heilbron (1936) and Bock and Wetter (1938) studied the sterols of invertebrates and found that the provitamin D content in the non-saponifiable matter was, on the average, higher than in vertebrates. Bock and Wetter were unable to detect cholesterol in mussels, but isolated a sterol not identical but isomeric with stigmasterol.

This investigation was concerned with the sterol fraction obtained from the mussel, *Mytilus planulatus* (Lamarck), Gabriel (1936).

Cholesterol and Provitamin D.

In the initial stages of the work the lipoid fraction was obtained by an acetone and ether extraction of the fresh material. Saponification yielded a crude sterol fraction. The alcoholic and non-alcoholic moieties were separated by the preparation of the acid-phthalic-acid esters, from which the free alcohols were obtained by saponification, acetylated and separated into three portions by fractional crystallization.

TABLE 1.

Material.	Source.	Time of collection.	Provitamin D content in p.c. of non-saponifiable matter. U.V. Absorption.	Rosenheim Reaction.
Mytilus planulatus (Lamarck), (Mussel)	Port Melbourne	July	7·3	8·0
" " " "	" "	October	5·0	3·5
" " " "	" "	January	—	5·0
" " " "	" "	April	—	<2·0*
Oysters	Stewart Isls.	September	12·5	18·0
Cardium tenuicostatum (Cockle)	Rye, Port Philip Bay	October	3·6–4·6	—
Scallop	Bought frozen at market	November	6·5	—
Sea Anemone	Sorrento, Port Philip Bay	March	0·9	—

The most soluble portion (M.P. 103–105°) had a molecular weight of 425, that of the middle fraction (M.P. 105–107°) 426, and that of the remainder, representing the bulk of the material (M.P. 122–124°) 435. The last fraction, following saponification, was completely precipitable by digitonin, indicating that it consisted solely of 3-β-OH sterols. The presence of 1·4 to 1·5 double bonds in each fraction was indicated by perbenzoic acid titration. This associated with the molecular weights suggested the presence of cholesterol. Accordingly, the acetates of each fraction were saponified. Following bromination in acetic acid, a colourless sterol dibromide (M.P. 109°), crystallizing in needles, was isolated. This compound, following debromination and purification, yielded a substance indistinguishable in its physical properties from cholesterol and constituted 25 p.c. of the total sterol fraction. The presence of cholesterol is subject to variation, and occasionally, in other batches of mussels, it could not be detected. In later work, the mussels were treated by the following procedure:

Following digestion with alkali they were refluxed with 95 p.c. alcohol and the resultant solution extracted with benzene. The residual matter was free of esters. The acetates prepared

from the residue yielded insoluble bromides which, however, were less stable than that obtained by the acetone-ether extraction. Only occasionally were crystalline bromides obtained, having a melting point identical with that of cholesteryl acetate dibromide. The mother liquor, freed from ether, turned olive green. This suggested the presence of a provitamin D. The Rosenheim reaction for these latter compounds was strongly positive; a rough quantitative estimation with trichloracetic acid indicated the presence of 5 p.c. provitamin D in the crude non-saponifiable matter, and approximately the same value was obtained by the ultra-violet absorption spectrum. The acetates from the non-saponifiable matter contained provitamin D in the least soluble fraction. The U.V. absorption spectra of this fraction and of the free sterols obtained following saponification are shown in Fig. 1.

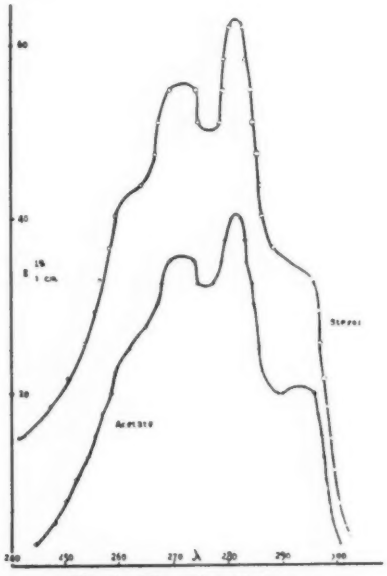

Fig. 1. Wave length in mμ.

The provitamin D content of mussels and other invertebrates is shown in Table 1. (Calculations made on the assumption that $E\frac{1\text{ p.c.}}{1\text{ cm.}}$ (281.5 mμ) = 320 for 100 p.c. provitamin D.)

The sterols prepared by the acetone-ether extraction (marked with asterisk) contained much less provitamin D as judged by the Rosenheim reaction. The long extraction period necessary for the complete removal of the lipoid matter from the mussels would, in all probability, lead to destruction of the provitamin D.

The Rosenheim reaction and the U.V. absorption spectrum (Schoenheimer and Evans, 1936) are not specific for any particular provitamin D (Hogness *et al.* 1937), but merely indicate the presence of a conjugated double bond Δ5–6, 7–8, in Ring B. The provitamin D in the mussels is probably not ergosterol (provitamin D_2) since neither the sterol mixture nor the acetates yielded "Ergopinacon" by radiation with sunlight in the presence of eosin (Windaus and Bock, 1937). No quantitative separation could be obtained by absorption of the sterol mixture on Al_2O_3 in the Tswett column although the highest concentration of provitamin D was found in the upper portions.

The free sterols obtained from several batches were combined, brominated in order to remove the insoluble cholesterol dibromide, the mother liquor debrominated with zinc dust and then acetylated. The purified acetates had a molecular weight of 441. Following bromination, a compound, crystallizing in plates and having a M.P. of 176°, was obtained. An additional compound was isolated from the mother liquor which crystallized in fine rods and had a M.P. of 170°. On analysis, both these compounds were shown to be tetrabrom derivatives. Since the melting point for tetrabrom ostreasteryl acetate is 150·5° (Bergmann, 1934b) and the corresponding stigmasterol derivative 211–212° (Windaus and Hauth, 1906), it appears that these compounds are not identical with either ostreasterol or stigmasterol.

Identification of Sterols Isolated from the Non-saponifiable Matter.

Method A. Freshly collected mussels were steamed for half an hour, freed from shells and dehydrated with 3 successive lots of acetone, and extracted with ether. The acetone and ether extracts were evaporated in a CO_2 atmosphere and the residue dissolved in acetone, filtered and evaporated. The resultant oil was saponified with 20 p.c. alcoholic potash, diluted with water and extracted with ether. The ether layer was washed with 5 p.c. KOH and then with water till neutral. Excess water was removed by standing over anhydrous Na_2SO_4 and then the ether distilled off. The residue, a pink-coloured semi-crystalline mass, constituted 0·2–0·3 p.c. of the minced tissue. 13·5 gm. of the non-saponifiable matter were dissolved in 65 gm. water-free pyridine and, after addition of 26 gm. phthalic-acid-anhydride, refluxed for 2 hours. After cooling to room temperature, the reaction mixture was made alkaline with Na_2CO_3 solution and extracted with ether. 2·5 gm. of a dark brown oil, representing the non-alcoholic portion, were obtained from the extract. The aqueous layer, after acidification, was extracted with ether, 25 gm. KOH in 100 ml. absolute alcohol were added, the ether distilled off and the remainder saponified for 2 hours. Extraction with ether and evaporation yielded 9·1 gm. of crude sterols. These were refluxed for 1 hour with 20 ml. acetic anhydride. 8·2 gm. of crude acetates were obtained. Recrystallization from absolute alcohol was repeated 4 times. 5·4 gm. of colourless plates, having a M.P. of 122–124°, were obtained. The M.P. remained constant in spite of further recrystallization.

The molecular weight (mean of 3 determinations) was found to be 435 by the method of Sandqvist and Bengtsson (1931), and a similar value was obtained by the technique of Fantl (1941), (2 determinations).

The oxygen consumed in a perbenzoic acid titration, after 27 hours reaction time at 4° C., showed the presence of 1·49 double bonds. Taking ergosterol as the standard, the provitamin D content was less than 2 p.c. when estimated by the Rosenheim reaction. From the mother liquor, from which the acetate (M.P. 122–124°) separated, two crystalline fractions were collected. The first amounted to 0·2 gm. (M.P. of 103–105°), had a molecular weight of 425 (Fantl) and contained approximately 2 p.c. provitamin D. The second fraction, weighing 1·6 gm. had a molecular weight of 426 and contained 1·46 double bonds. 120 mg. of a dibromide were obtained from 170 mg. of a sterol isolated from this fraction after saponification following bromination in glacial acetic acid and ether. Debromination with NaI, after Schoenheimer (1935), yielded a sterol, M.P. 144°.

The fraction (M.P. 122–124°) was saponified and a sterol mixture, M.P. 135–137°, collected. 2·2 gm. dissolved in ether gave, with 32 ml. 5 p.c. bromine-acetic acid solution, 0·61 gm. of white needles, M.P. 109°. 0·368 gm. of the bromide was refluxed with NaI in alcohol; 12·5 ml. N/10 thiosulphate were necessary for titration of I_2 liberated. (Calculated for $C_{27}H_{46}OBr_2$ 13·5 ml.) The isolated sterol crystallized from alcohol in plates and in needles from acetone, and had a M.P. of 146–147° which was unchanged after addition of an authentic sample of cholesterol. (α) $D^{17} = -38·6°$.

The filtrate from cholesterol dibromide, after removal of the ether, was filtered in the cold and refluxed with zinc dust for 3 hours. 0·81 gm. sterol was obtained which, following acetylation, was crystallized from alcohol and had a M.P. of 132–134°, and a molecular weight of 441 (2 determinations). Bromination of this product in the cold gave a colourless bromide in 50 p.c. yield having a M.P. of 171–172°. The compound was purified by precipitation with ethyl alcohol from ethyl-acetate solution. Its bromine content was 40·67 p.c. (Micro-Carius), and hence it can be identified with a stigmasterol isomer $C_{31}H_{50}O_2Br_4$ having a calculated bromine content of 41·30 p.c., rather than with a dehydrocholesterol derivative $C_{29}H_{50}O_2Br_4$ having a bromine content of 42·85 p.c.

Method B. The digestion of mussels, and other invertebrates investigated, was carried out by allowing them to stand at room temperature over night in 12 p.c. KOH. 1·2 vol. of methylated spirit were added and the solution refluxed for 2 hours. The hydrolysate was filtered, an equal volume of water added and extraction with benzene carried out. The combined extracts were washed with 5 p.c. KOH, dried over Na_2SO_4, the benzene distilled off in a CO_2 atmosphere, and the residue dissolved in acetone, filtered and evaporated. The residue was free from esters. The crude sterols were acetylated and repeatedly recrystallized from absolute alcohol.

Properties of *sterol acetates* from mussels.

Time of year collected.	M.P.	Molecular weight.	Double bonds.	Cholesterol content.
April	120–24°	—	—	present, identified as acetate
July	122–24°	436	1·7	„ „ „ dibrom-acetate
November	—	433	1·68	not tested
December	—	—	—	absent
January	—	433	1·62	not tested

The acetates from the different batches were combined, saponified and the free sterols brominated in order to remove cholesterol dibromide. Following evaporation of ether in the

cold, a second crop of cholesterol dibromide crystals was deposited. The mother liquor was debrominated with zinc dust and the isolated sterols acetylated and brominated. From 0·94 gm. acetates, 0·3 gm. of a bromide, M.P. 161–162°, was obtained. After dissolving in warm ethyl acetate and precipitating with alcohol, a substance, crystallizing in plates and having a M.P. of 176°, was isolated. The bromine content was 41·45 p.c. The mother liquor of the bromide was freed from ether whereupon 0·6 gm. of a bromide deposited. This was only slightly soluble in ethyl acetate and absolute ethyl alcohol, had a M.P. 170°, and a bromine content of 41·58 p.c.

SUMMARY.

The salt water bivalve, *Mytilus planulatus* (Lamarck), has frequently been found to contain cholesterol. Compared with other food stuffs, the bivalves investigated are the richest source of provitamin D. Further, the mussels contained unsaturated sterols having two double bonds. Two compounds were isolated as tetrabrom acetates and were not identical with the corresponding derivatives of ostreasterol and stigmasterol. The presence of sterols, having a lower molecular weight than stigmasterol, is indicated by molecular weight determinations on the isolated acetates.

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SEROLOGICAL STUDIES ON PLANT VIRUSES

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INTRODUCTION.

It is now well known that many plant viruses can act as antigens. The mixing of antigen and antibody is followed by a number of reactions which may be observed *in vitro*. Of these the precipitin reaction has been the most used in serological research on plant viruses. By the use of this test it has been possible to obtain data on the relationships of certain viruses and to determine their concentration.

The precipitin test was first successfully applied in plant virus work by Dvorak (1927). Beale-Purdy in a series of papers (1929, 1931, 1931a, 1934) clearly demonstrated the antigenicity of tobacco mosaic virus, and showed that sap from infected plants contained, in addition to the antigenic substance of the healthy plant, a further specific fraction due to the virus. Birkeland (1934, 1935), Gratia (1934), and Chester (1935, 1935a, 1936) extended the precipitin technique to the study of many viruses and obtained further evidence on the specificity of serological reactions. Chester (1937) developed a field method for the rapid identification of plant viruses. Dunin and Popova (1937) also described a drop method of precipitin testing which could be used in the field.

This paper describes the application of the laboratory and field precipitin techniques to the study of a number of plant viruses, and discusses the question of their relationship as shown by their antibody production.

EXPERIMENTAL METHODS.

The technique used by Chester (1935) was followed with some modifications. Preliminary tests to obtain the optimum antigen-antibody ratio were applied.

Plant virus material. The following viruses were tested serologically in the course of this investigation:

Virus.	Source.
* Ordinary tobacco mosaic (*Marmor tabaci* var. *vulgare* H.)	B. Grieve (Melbourne)
* Tobacco aucuba (*Marmor tabaci* var. *aucuba* H.)	J. Johnson (Wisconsin)
* Tomato bushy stunt (*Marmor dodecahedron* H.)	K. Smith (Cambridge)
* Potato virus X (*Marmor dubium* var. *vulgare* H.)	J. G. Bald (Canberra).
* Potato viruses X + B (Bawden)	" " " "
* Potato aucuba mosaic (*Marmor aucuba* H.)	" " " "
* Tuber blotch (Loughnane and Clinch)	" " " "
Potato spindle tuber	" " " "
* Tomato spotted wilt (*Lethum australiense* var. *typicum* H.)	Collected by author
Rose wilt (*Marmor flaccumfaciens* H.)	Collected by author
Strawberry crinkle (*Marmor fragariae* H.)	S. Fish (Burnley)

Viruses marked with an asterisk were propagated in tomato plants, while the others were propagated in their natural host plants.

Most of the viruses were propagated in plants grown in an insect proof glasshouse. Purity of virus was tested by the symptom picture in differential host plants. In the case of rose wilt virus and strawberry crinkle virus, field material was used since these plants were found to harbour only the required virus.

Preparation for antisera. Rabbits were used as test animals for production of antisera. Sap from infected plants was strained through cheesecloth, centrifuged for 30 minutes at 3,000 r.p.m., and 0·25 ml. of 17 p.c. NaCl solution was added per 5 ml. of juice to obtain 0·85 p.c. concentration. Injection of sap into rabbits and subsequent operations followed the usual procedure.

To test a precipitin strength of serum a sample of blood was taken from the ear-vein of a rabbit six days after the last inoculation. In order to obtain clearer sera, fasting of the animals

prior to bleeding, as recommended by Jordan and Falk (1928), was practised. When a satisfactory result was obtained, the rabbit was bled from the heart. At first the sera were preserved with phenol in 0·3 p.c. concentration; later merthiolate solution was used, as this gave a clearer serum. Serum was stored in a refrigerator at 0° C. Stable viruses gave sera which deteriorated only slightly over a period of four years, while sera obtained with less stable viruses were less potent and showed a progressive diminution leading to complete loss of precipitin titre after a few months' storage.

Preparation of test antigens. The saps from healthy and infected plants were expressed from young leaves, centrifuged and the sodium chloride concentration of the supernatant liquid was adjusted to 0·85 p.c. The method most often adopted was to freeze the juice overnight. Next day, after thawing out, the juice was centrifuged again, a part of the normal sap being then set aside for "absorption" (as described below), while the balance of the normal sap and the virus suspensions were left overnight in a refrigerator.

Immune serum obtained with virus sap contains antibodies against two antigens, viz. the normal and the virus proteins of the juice. The normal protein antibodies were removed by the "absorption" method and finally the virus suspensions and normal sap were centrifuged again to serve as antigens.

Precipitin tests. Preliminary tests were made to obtain a maximum flocculation reaction between the antigen and antibody. For this the reagents have to be present in certain definite proportions and suitable dilutions must be tested out since, as the "prozone" phenomenon shows, an excess of one of the reagents may interfere with the precipitin formation. Further the presence of an electrolyte is necessary for the reaction and normal saline solution is used. The titration was done by preparing in a set of test tubes progressive dilutions of antigen (virus juice) in normal saline ($\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ and $\frac{1}{16}$ dil.) and of immune serum (previously absorbed serum was already at $\frac{1}{3}$ dilution and further dilutions in saline were $\frac{1}{6}$, $\frac{1}{9}$ and $\frac{1}{12}$). Reagents at these dilutions in 0·2 ml. quantities (= 1 volume) were then distributed into small precipitin tubes so as to form a chessboard arrangement of the mixtures (see Table 1).

TABLE 1.

Titration of antigen-antibody ratio.

	Tube No. 1	2	3	4
	Dilutions of antibody.	Dilutions of antigen.		
Row 1	$\frac{1}{3}$ →	$\frac{1}{2}$ ↓	$\frac{1}{4}$ ↓	$\frac{1}{8}$ ↓	$\frac{1}{16}$ ↓
,, 2	$\frac{1}{6}$ →				
,, 3	$\frac{1}{9}$ →				
,, 4	$\frac{1}{12}$ →				

Thus in the horizontal row 1, tube 1 contains 1 vol. $\frac{1}{2}$ dil. antigen + 1 vol. $\frac{1}{3}$ dil. antibody. Control tubes of the lowest dilutions of antigen + saline, and of antibody + saline were provided. The tubes were placed in a water bath at 37° C. With the more stable viruses (e.g. tobacco mosaic or potato X virus) the first tube to show flocculation was usually tube 2 in row 2 (the optimum ratio being 3: 2). With the less stable viruses (e.g. tomato bushy stunt) the first tube to show flocculation was tube 1 in row 1 (optimum ratio 3: 2).

In precipitin tests one may either use geometrical progressive dilutions of antigen, keeping the immune serum at a constant concentration, or vice versa. Experiments performed to discover which method gave the better result showed a more abundant precipitate when antigen at different dilutions was added to antiserum at a constant concentration and so this latter procedure was adopted. In these experiments the antiserum was at $\frac{1}{6}$ dil. for the stable viruses and at $\frac{1}{3}$ dil. for the less stable ones.

Precipitin titrations were carried out according to the normal procedure and adequate controls were included. The mixtures were incubated at 37° C. for two hours, read, then left overnight in a cool room and read again. The titre of the serum was indicated by the highest dilution of the antigen precipitating with its antiserum.

EXPERIMENTAL RESULTS.

Table 2 shows that of the eleven viruses tested, seven gave positive precipitin reactions. Serological relationship was demonstrated to occur only in the case of the tobacco mosaic viruses, *vulgare* and *aucuba*.

The results obtained with the tobacco mosaic viruses, tomato bushy stunt and potato virus X were in general agreement with those obtained by Chester (1935), Bawden and Pirie (1938a, b) and Gratia and Manil (1934a).

Potato viruses. Of the five viruses tested, four gave precipitin reactions. Virus B was not available in pure culture but was present together with X virus. There were no cross reactions between the other potato viruses and so they are considered to be serologically unrelated. The juice of potato tubers proved to be unsatisfactory as antigen.

TABLE 2.

Precipitin reactions of various plant viruses.

Immune serum (from immunized rabbits).

Test antigens (clarified virus containing sap).	Tobacco mosaic *vulgare*.	Tobacco mosaic *aucuba*.	Tomato bushy stunt.	Potato virus X.	Potato viruses X + B.	Potato aucuba mosaic.	Tuber blotch.	Potato spindle tuber.	Tomato spotted wilt.	Rose wilt.	Strawberry crinkle.
1 Tobacco mosaic *vulgare*	1/64 (9IP inj.) 1/512 (12IP inj.)	–	0	0	0	0	0	–	–	–	–
2 Tobacco mosaic *aucuba*	1/64 (12IP inj.) –	1/32 (9IP inj.) 1/256 (12IP inj.)	0	0	0	0	0	–	–	–	–
3 Tomato bushy stunt	0	0	1/16	0	0	–	–	–	–	–	–
4 Potato virus X	0	0	0	1/128	–	0	0	–	–	–	–
5 Potato viruses X + B	0	0	0	–	1/256	0	0	–	–	–	–
6 Potato aucuba mosaic	0	0	–	0	0	1/16	0	–	–	–	–
7 Tuber blotch	0	0	–	0	0	–	1/4	–	–	–	–
8 Potato spindle tuber	–	–	–	–	–	–	–	0	–	–	–
9 Tomato spotted wilt	–	–	–	–	–	–	–	–	0	–	–
10 Rose wilt	–	–	–	–	–	–	–	–	–	0	–
11 Strawberry crinkle	–	–	–	–	–	–	–	–	–	–	0

Potato X + B viruses. The precipitin titre of the virus complex X + B was 1/256. No record was found in the literature of the antigenic character of potato virus B. A serological reaction was obtained also with material believed to harbour pure B virus. A precipitin test was then performed in which virus complex X + B was set up against X + B antiserum absorbed with B virus material, controls being provided to ensure complete absorption of the B fraction. A precipitin reaction was obtained. But when a similar test was set up a few weeks later no flocculation appeared. Soon afterwards the plants exhibited symptoms of X virus infection. This experiment is interesting in two respects: (1) X and B viruses, although they may be serologically related, have a different antigenic structure, as shown in the first test, X fraction not being absorbed with B, and (2) the sensitivity of the serological reaction.

As no pure X virus material was available at the time of this experiment, absorption of X fraction with the object of obtaining pure B virus could not be attempted.

Potato aucuba mosaic virus. The titre obtained for this virus was 1/16, being in agreement with the result obtained by Chester (1935).

Tuber blotch virus. The precipitin titre obtained was 1/4. No record was found in the literature of the serological properties of this virus.

Potato spindle tuber virus. No serological reaction was obtained as was also recorded by Chester (1937).

No precipitin reactions were obtained in all experiments with tomato spotted wilt, rose wilt and strawberry crinkle viruses although a variety of procedures was tried.

Tomato spotted wilt virus. Absence of precipitin reaction was also recorded by Chester (1937). T.S.W. has been shown to inactivate soon at room temperature unless a reducing agent, e.g. sodium sulphite is added to the virus-containing juice (Bald and Samuel, 1934). Therefore, to prolong the longevity of the virus, the juice used for the injections and for the antigen reagent was mixed with an equal volume of 0·2 p.c. sodium sulphite. Using tomato and arum lily infected plants as source of virus, clear antigens were obtained. When Tropœolum majus juice was used (this plant harbours only the virus of T.S.W.) some points were noted: (1) A chessboard arrangement of antigen and antibody indicated that both normal, and virus-infected

sap autoprecipitated with normal saline in low dilutions (½ and ¼), while serum in dilution ⅓ and ⅙ arrested their precipitin formation; and (2) the addition of normal saline to normal sap gave more precipitate than when added to juice containing the virus.

Attempts to get clear antigens from Tropœolum majus sap by two methods involving adjustment of pH to 7·6 and freezing and thawing were unsuccessful.

Rose wilt virus. This virus is believed to be unstable (Grieve, unpublished data). Work on this virus was continued from preliminary experiments by Prof. Woodruff and Dr. Grieve. No reference has been found in the literature concerning its antigenic character. Prolonged injections were tried in an endeavour to stimulate antibody production but without success. It was found that antiserum had to be mixed with healthy sap in proportion 1: 6 to get complete absorption of normal protein antibodies.

Strawberry crinkle virus. No reference to serological reactions of this virus was found in the literature.

The antigens used for the precipitin tests were concentrated *in vacuo*. It was noticed that the sap from older shoots, unless well centrifuged, autoprecipitated readily with normal saline. Very young shoots had therefore to be used. It is interesting to note that no precipitate was obtained with the normal strawberry sap and non-absorbed antiserum.

Virus Concentration in Different Plant Parts as shown by the Precipitin Reactions.

Precipitin titres of tobacco mosaic aucuba and of potato virus X in different parts of tomato plants indicated that the highest concentration of both viruses occurred in leaves and the lowest in stems and petioles. The results for tobacco mosaic aucuba are in agreement with those of Matsumoto and Somazowa (1932).

Observations on the Character of the Precipitates in Precipitin Reactions.

In the course of this work it was noticed that different viruses gave characteristic types of precipitates. Tobacco mosaic viruses gave an abundant precipitate, light in texture; the precipitate of potato virus X was still lighter in texture but less abundant; the floccules of potato aucuba mosaic were also light in texture but rather coarser than that of potato virus X; while the particles of tomato bushy stunt virus precipitate were tightly grouped together. Bawden and Pirie (1938) considered tomato bushy stunt virus floccules to be similar to those obtained with bacterial "O" antigens, and the tobacco mosaic and potato virus X precipitates to resemble the type given by "H" antigens. The author is in agreement with these views and also considers that potato aucuba mosaic virus could be included in the second group.

APPLICATION OF FIELD METHODS.

The value of field methods, i.e. simplified precipitin tests in identifying plant viruses has been stressed by Chester (1937) and by Dunin and Popova (1937). Miller (1938), and Valleau and Diachun (1938) however obtained some contradictory results with Chester's method, so that it was considered of value to test out the field method technique on the viruses under survey.

Chester's field method. The procedure consists of mixing crude juice, freshly expressed from a virus infected plant, with its antiserum at optimal dilution. Later (1937a) Chester recommended the centrifuging of the juice preliminary to its use.

Experience with Chester's field method soon made it evident that a preliminary test to determine the optimum ratio of flocculation had to be set up. In the case of stable viruses, e.g. tobacco mosaic or potato virus X the optimum ratio usually proved to be with antigen in ¼ and antiserum in ⅛ dilution. With viruses producing weaker antisera the quickest reaction was usually obtained when antigen was in ½ and antiserum in ⅓ dil. The optimum ratio in the field method was in general agreement with the one obtained by the laboratory flocculation test.

When the best experimental conditions for the field method had been worked out, the eleven viruses, used in the course of this work, were tested. Results in close agreement with those obtained by the application of the laboratory flocculation technique were found for the viruses which had a high precipitin titre. For those with a low precipitin titre, e.g. potato tuber blotch, the field method was less convincing.

Dunin and Popova's drop method. The procedure is to mix a drop of freshly expressed virus-containing juice with a drop of antiserum on a slide. A positive test is indicated by a precipitate forming almost immediately in the drop of liquid.

In the course of this work three viruses were tested, viz.: tobacco aucuba, potato virus X and potato aucuba mosaic. While in many cases the reactions were satisfactory, some artifacts were encountered, so that controls with normal serum sometimes showed flocculation. The virus + antiserum gave an almost immediate reaction, while the control with normal serum showed a lag of a few seconds before flocculation took place but the time factor was too limited to be considered reliable. In hot weather and with juice from older plants the precipitation with normal serum took place even more readily. Sometimes artifacts could be avoided by collecting the

crude juice into a small tube and letting it stand for a while till the gross particles had settled down. The positive reaction was then less spectacular but the controls with normal serum remained clear.

The author is of the opinion that the drop method is not reliable for positive reactions because of artifacts. On the other hand a clear cut negative result is dependable.

SUMMARY.

Using the precipitin titration technique a serological study has been made on a number of plant viruses.

Positive flocculation reactions were obtained with the following viruses: ordinary tobacco mosiac, tobacco aucuba, tomato bushy stunt, potato virus X, potato viruses X + B, potato aucuba mosaic and potato tuber blotch; while the viruses of potato spindle tuber, tomato spotted wilt, rose wilt and strawberry crinkle gave negative results.

Cross precipitin tests showed that of the viruses tested only the two strains of tobacco mosiac virus were serologically related.

Investigations on the field methods of precipitin testing showed that Chester's method gave reliable results when the optimum antigen-antibody flocculation ratio was applied, while Dunin and Popova's drop method was not found to give consistent results.

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INTRACORPUSCULAR BILE PIGMENT FORMATION

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The formation of bile pigment from haemoglobin is known to occur in the cells of the reticulo-endothelial system, mainly in the liver, bone-marrow and spleen, but also in blood extravasations, e.g. pathologically in pleural fluid and physiologically in large amounts in the dog's placenta. Intravascular formation has occasionally been assumed but without stringent proof. More recently Barkan *et al.* (1937, 1938) have come to the conclusion that part of the bile pigment is formed in the circulating red cell. Their evidence is mostly indirect, based on the interpretation of the "easily detachable" erythrocyte iron as bile pigment-iron-protein complex. Legge and Lemberg (1941) have shown that the greater part of the "easily detachable iron" is derived from haemoglobin, but Barkan's hypothesis was partly confirmed by the isolation of small amounts of biliverdin, derived from a bile pigment-iron-protein, both from the erythrocytes of various animals and from crystalline horse oxyhaemoglobin. Barkan and Walker (1939, 1940) have tried to show that this first step of the bile pigment formation occurs in the circulating red cell and determines its life time. The experiments of Burmester (1937) and particularly those of Miller and Hahn (1940) with radioactive iron are, however, in discord with this assumption. The bile pigment-iron complex may be a relic from the time when the immature red cell in the bone marrow still possessed a nucleus and a strong respiration.

In the present paper we offer the first direct proof for the formation of bile pigment in the circulating red cell. Phenylhydrazine is a drug well known to cause a strong bile pigment formation, which in many aspects appears to be closely related to the physiological process and is used for the destruction of red cells in polycythaemia vera. Large amounts of free biliverdin as well as of choleglobin are found in rabbits' erythrocytes resulting from the action of phenylhydrazine in vivo. The drug does not therefore cause the bile pigment formation by haemolytic action.

We report also a few quantitative determinations of the biliverdin yield from erythrocytes of normal and pathological human blood.

EXPERIMENTAL.

Isolation of Biliverdin from Red Cells.

The blood was oxalated and red cells and plasma separated by centrifugation and washing with physiological saline.

Before treatment with acetic acid in the manner described in principle by Lemberg, Lockwood and Legge (1941b), the cells must be laked and diluted to a haemoglobin concentration of less than 5 p.c.; otherwise no homogeneous solution is obtained.

A volume of the cells is measured in 1 or 2 c.c. pipettes ("to contain"), the outside of the pipettes wiped off and the content blown into nine volumes of distilled water saturated with ether. The solution is repeatedly sucked up into the pipette until the haemolysis is complete. The haemoglobin content of the laked blood is measured spectrophotometrically by diluting to a concentration of 0·1–0·2 p.c. Hb. with 0·1 p.c. sodium carbonate solution and measuring at 578 mμ in a 2 cm. or 1 cm. tube with a Hilger-Nutting-Barfit spectrophotometer. The specific extinction coefficient (defined as extinction coefficient of a 0·1 p.c. solution in a layer of 1 cm. depth) of oxyhaemoglobin at 578 mμ, was found to be 0·95. The presence of small amounts

[1] This work was carried out under a grant from the National Health and Medical Research Council.

of oxygenated choleglobin would not cause any appreciable errors in the haemoglobin determination.

For the extraction of biliverdin 10 mg. of ascorbic acid are added to the laked cells, followed immediately by twice the volume of acetic acid. The addition of ascorbic acid prevents the transformation of biliverdin into biliviolin (cf. Lemberg *et al.* 1941b). This 66 p.c. acetic acid solution of "acid haematin" is allowed to stand for 15 minutes at room temperature, before the main bulk of the proteins is precipitated by the double volume of peroxide-free ether. After some standing the protein is filtered off. The ethereal solution is then washed with sodium acetate solution and the washings treated as described in the paper, quoted above, on p. 369. A precipitate of haemin coming out during the final washing is removed by filtration, if it becomes so bulky as to prevent the separation of the layers. Finally the ether solution is extracted in a small separating tube, once with 2 c.c. and then repeatedly with 0·5 c.c. of 20 p.c. hydrochloric acid, until the final volume is about 9 c.c. The acid extracts are collected in a 10 c.c. measuring cylinder. At this stage the formation of a small amount of haemin precipitate and emulsification at the interface can not usually be avoided and small amounts of the emulsion (about 2 c.c.) are collected with the acid phase. After standing over night the volume of the aqueous phase is read, the top layer of ethereal haemin solution removed with a Pasteur pipette and the acid solution filtered through a small thin filter paper (Munktell's No. 1F). The bile pigment content of the clear green to blue solution is then determined spectrophotometrically with method 3 (cf. Lemberg *et al.* 1941b, p. 370).

Determination of Choleglobin.

The method used was essentially that described by Lemberg *et al.* 1941a. After prolonged action of phenylhydrazine the erythrocytes contain denatured globin which even in the alkaline solution (1 p.c. NaOH) in which the absorption of the CO-haemochromogens is measured, does not dissolve to perfectly clear solutions. In this case the ratio R = ϵ630 mμ: ϵ570 mμ gives too high values for choleglobin, since the cloudiness adds to the absorptions at both wavelengths to the same extent. This error is avoided by measuring also ϵ740 mμ and subtracting it from ϵ630 mμ and ϵ570 mμ. At 740 mμ neither haemoglobin nor choleglobin absorb light strongly, but the absorption of the latter cannot be neglected. A new formula, based on the measurements of the extinction coefficients of CO-protohaemochromogen and CO-cholehaemochromogen at 740, 630 and 570 mμ (Table 1) was therefore worked out, which allows the calculation of the ratio of choleglobin : haemoglobin (x) from the observed ratio $R' = \frac{\epsilon 630 - \epsilon 740}{\epsilon 570 - \epsilon 740}$. $x = \frac{R' - 0{\cdot}029}{0{\cdot}71 - 0{\cdot}17R'}$ (1). In the laked blood obtained after short action of phenylhydrazine this cloudiness is hardly noticeable and the results obtained by the formula $x = \frac{R - 0{\cdot}035}{1 - 0{\cdot}45R}$ (2) agree well with those obtained by the new formula from R′ (e.g. after 2 hrs. x found from R 0·083, from R′ 0·075). In the later stages, however, the deviation becomes large, e.g. after 21 hrs. x found from R 0·23, from R′ 0·127. While in the formula on p. 342 (Lemberg *et al.* 1941a) choleglobin is determined in per cent. of the sum of choleglobin and haemoglobin, which in these experiments was known from the original haemoglobin concentration, x in the formulae (1) and (2) means the ratio of choleglobin to haemoglobin.

TABLE 1.

	Esp.	740 mμ	630 mμ	570 mμ
CO-protohaemochomogen:		0·005	0·030	0·86
CO-cholehaemochromogen:		0·25	0·86	0·39

Action of Phenylhydrazine in Vivo.

A rabbit of 2·5 kg. weight was injected with a neutralized solution of 50 mg. phenylhydrazine hydrochloride per kg. Blood was taken at various intervals and its haemoglobin and choleglobin content and the yield of biliverdin obtained from the erythrocytes determined as described above. The results are given in Table 2.

TABLE 2.

Hrs. after injection of phenylhydrazine.	Haemoglobin in cells in p.c.	R′	Choleglobin (100x).	Bile pigment, μg. per 1 c.c. of cells: calculated from choleglobin.	Bile pigment, μg. per 1 c.c. of cells: found.
0	—	0·030	(0)	(0)	3–8
2	30	0·081	7·5	43	36
4	26	0·086	8·2	42	117
21	24·3	0·117	12·7	60	66
46	24·2	0·109	11·8	55	62
150	19·8	0·036	1·0	4	10

The last column shows that considerable amounts of bile pigment are obtained from the cells. At the maximum, 4 hours after injection of phenylhydrazine, a concentration of 11·7 mg. bile pigment per 100 c.c. of cells is reached, the greater part of which consists of biliverdin, the remainder of biliviolin. This concentration is of the order of size of the highest bilirubin concentrations in plasma observed in severe jaundice. It is to be noted that little more than 50 p.c. of biliverdin added to red cells can be recovered by our procedure (cf. Lemberg *et al.* 1941b) and that the real bile pigment concentration in the cells is therefore about twice as high. If the effect of phenylhydrazine in man is the same, this would mean that during 4 hours 470 mg. of bile pigment have been formed inside the corpuscles. This is more than the normal daily bile pigment formation, generally estimated as 400 mg. per day. There can thus be no doubt that a large part of the bile pigment obtained by the action of phenylhydrazine is formed inside the corpuscles.

A comparison of the amount of bile pigment isolated from the cells with that which can be derived from choleglobin, given in the preceding column, shows that at the stage of the maximum bile pigment formation the greater part is present as free biliverdin, while initially all is derived from choleglobin. The figures in this column have been calculated on the assumption (cf. Lemberg *et al.* 1941b) that 11 p.c. of choleglobin is split to bile pigment and 50 p.c. of this is recovered from the cells; they are thus directly comparable with those of the last column. Phenylhydrazine *in vivo* acts thus in the same maner as ascorbic acid *in vitro*, forming first choleglobin and later free biliverdin. In the later stages the haemoglobin content of the cells still appears to drop[2] and the choleglobin content rises slightly, while the total bile pigment drops to a figure indicating no large excess over that derived from choleglobin. This is to be explained either by a rapid destruction of the cells which contain free bile pigment or by a more rapid removal of bile pigment from the cells and transformation into plasma bilirubin.

Isolation of Biliverdin from Human Erythrocytes.

In our previous paper we had reported sheep erythrocytes to yield between 3 and 7·5 μg. of biliverdin per 1 c.c., while 1 c.c. of a 15 p.c. solution of crystalline horse oxyhaemoglobin gave 2–4 μg.

The figures now obtained for normal human blood are of the same order. They varied between 0·4 and 2 μg. per c.c. of blood and between 1 and 5 μg. per 1 c.c. of cells. In normal placental blood, which contained 6 μg. of bilirubin in 1 c.c. of plasma, we found in the cells 3·3 μg. of biliverdin.

Blood of an untreated case of pernicious anaemia was also investigated. The patient was a man of 57. Haemoglobin was 5·74 gm. per 100 c.c., erythrocytes 1,500,000 with a colour index of 1·3 and macrocytosis and poikilocytosis. Reticulocytes: 1 p.c. Bilirubin in serum (Thannhauser modification of van der Bergh method) was 1·0 mg. per 100 c.c. 1 c.c. of cells yielded 8·3 μg. biliverdin, just slightly higher than found in normal blood. This is hardly significant, particularly since the colour index was high. In such cases it is best for comparison to calculate the yield corresponding to 1 c.c. of a 15 p.c. solution of haemoglobin. This (3·75 μg. per c.c.) falls into the normal range.

Only in one case have we so far discovered a pathological intracorpuscular bile pigment formation. This was in a newborn male baby dying a few hours after birth with erythroblastosis foetalis without clinical icterus. Blood was taken 2 hours after birth, shortly before death occurred. The blood contained 19·3 gm. of haemoglobin per 100 c.c. and no choleglobin ($R' = 0·025$ in laked cells). 1 c.c. of this blood yielded 23 μg. of biliverdin, about 7 times as much as normal placental blood.

SUMMARY.

By the injection of phenylhydrazine into rabbits the choleglobin and biliverdin content of their erythrocytes is increased. This intracorpuscular bile pigment formation accounts for a large part of the bile pigment formation caused by the drug.

Normal human blood yields small amounts of biliverdin (a few μg. per c.c.). This was found not significantly higher in a case of untreated pernicious anaemia, but much higher in a case of erythroblastosis foetalis.

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[2] We have not done haematocrit readings so that "haemoglobin content of the cells" means only haemoglobin content of the packed cells as used for biliverdin and choleglobin determinations, not in the exact sense.

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A CONTRIBUTION TO THE PHARMACOLOGY OF YOHIMBINE

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The ability of yohimbine to produce vaso-motor reversal and the fact that it can be used to replace ergotoxine in this respect have been reported by Raymond-Hamet (1935). Cannon and Rosenblueth (1935) have used it in this connection in their experimental work. The literature, however, contains no comment on certain points such as the effects of anaesthesia or of different anaesthetics on this action. These are important in view of the finding of Herwick, Linegar and Koppanyi (1939) that the vaso-motor reversal effect of ergotoxine is not obtained in the presence of nembutal.

This paper reports a confirmation of the general findings, an investigation of these special aspects and contains additional notes on the pharmacological action of yohimbine on other organs.

EXPERIMENTAL WORK.

The Arterial Blood Pressure of the Cat.

(A) The action of yohimbine on the vaso-motor response to adrenaline was studied on cats: (a) anaesthetized with chloroform, ether, dial or nembutal; (b) decerebrated; (c) decapitated.

In one set of experiments, the cat was first anaesthetized, then decerebrated, and later decapitated, the vaso-motor reversal action being examined at each stage. In a second set of experiments, the cat was either decerebrated or decapitated and the vaso-motor reversal action studied at only the one stage; in a third set, any two of the above procedures were combined.

The blood pressure was recorded from the carotid artery. Adrenaline and yohimbine were injected into the saphenous vein. The adrenaline dosage sufficient to give a clear cut effect on the blood pressure was found by trial.

Yohimbine 1 mg./kg. was then injected and after an interval of from 8 to 20 minutes the original dose of adrenaline was again injected.

The following results were obtained: In nearly all cases in groups (a) and (b) yohimbine produced reversal of the adrenaline rise. On the animals in group (c), however, the reversal was frequently replaced by either a smaller rise in blood pressure or abolition of any rise. This effect was also noted in those animals in groups (a) and (b) which did not show the vaso-motor reversal. A comparison of the results in (c), in which a vaso-motor reversal was not obtained, with the few in (a) and (b) in which the reversal was also absent led us to the belief that the reversal is obtained only if the blood pressure is above 55 mm. of mercury.

Fig. 1. *Blood pressure of decerebrate cat.* Time: 30 secs.; AD. = adrenaline, 0·1 c.c. 1: 25,000; YO = yohimbine, 1 mg./kg.

In no case was yohimbine (in the dosage used) without any effect on the adrenaline response.

It should be noted that in groups (a) and (b) yohimbine usually produced a transient fall in blood pressure but, after approximately three minutes, the original level was restored. The effect of yohimbine persisted for 1–2 hours, that is to say a further injection of adrenaline within this period produced a fall in blood pressure.

In several experiments cocaine was injected after the first vaso-motor reversal and this potentiated the subsequent fall given by a second dose of adrenaline.

The results of a typical experiment are shown in Fig. 1.

(B) We then investigated in a similar manner, the effect of yohimbine on the blood pressure rise produced by ephedrine and neosynephrine.

The results obtained showed that yohimbine, again approximately 1 mg./kg., lessened but did not reverse the vaso-motor response of the cat to both these drugs.

The Action of Yohimbine on the Response of Other Tissues to Adrenaline.

(A) *The isolated frog's heart (Straub cannula).* The dose of adrenaline sufficient to produce a definite effect on the heart was obtained, this was washed out, yohimbine was then added to the perfusion fluid in the cannula and left for 5 minutes. After further washing, the second dose of adrenaline was added.

Varying concentrations of yohimbine were used and each was left in contact with the heart for approximately 5 minutes.

It was found that weaker concentrations of yohimbine, from 1: 10^7 up to 1: 10^6 had no apparent effect on the response of the heart to adrenaline, whereas stronger concentrations, e.g. 1: 10^5 stopped the heart beat and the results of the following doses of adrenaline were therefore not comparable with those of the initial dose.

(B) *The perfused frog's blood vessels (Trendelenburg's method).* The dose of adrenaline sufficient to produce vaso-constriction was found, yohimbine was then introduced into the perfusion fluid and it was found that if the following dose of adrenaline were given within 2 to 3 minutes of the yohimbine the vaso-constrictor action of adrenaline was abolished. If the time interval were longer yohimbine was ineffective.

Yohimbine was tested in concentrations of 0·5 c.c. of 1: 10^4 and 0·5 c.c. of 1: 10^5.

(C) *The isolated uterus of the rabbit and the rat.* Strips of uterus were placed in a bath of about 10 c.c. volume and kept at a constant temperature of 37° C. Adrenaline and yohimbine were added alternatively to the bath (0·1 c.c. each time). Yohimbine was left in the bath for 5 to 8 minutes before being washed out and replaced by the subsequent dose of adrenaline.

Yohimbine in concentrations of 1: 10^6 up to 1: 10^4 diminished the contraction produced in the rabbit's uterus by adrenaline.

Yohimbine in the concentrations tested appeared to have no effect on the response of the rat's uterus to adrenaline.

(D) *The isolated intestine of the rabbit, the rat, and the cat.* The volume of the bath was approximately 20 c.c. The rabbit's intestine was kept at 32° C., the rat and the cat intestine at 37° C.

In each case the dose of adrenaline sufficient to produce a marked relaxation was first obtained, yohimbine was then introduced into the bath and left for 10 minutes. This was washed out before the original dose of adrenaline was again added to the bath.

On the cat intestine, yohimbine in fairly strong concentration (0·1 c.c. of 1: 10^3) depressed the adrenaline response. On the rabbit intestine this concentration abolished the adrenaline response and in weaker concentrations (up to 1: 10^6) decreased it. With the rat intestine yohimbine itself produced such a prolonged relaxation that the interpretation of results was impossible.

DISCUSSION.

In the experiments on the blood pressure of the cat we found that, on those animals which had a blood pressure above the average for the particular type of experiment, yohimbine always produced reversal of the pressor action of adrenaline, even in the spinal cat, e.g. in 17 of the 20 experiments on anaesthetized and decerebrate animals, the blood pressure range was from 100 to 200 mm. mercury, and in these cases yohimbine produced vaso-motor reversal. In only 3 of the 20 was there no reversal and in these cases the blood pressure range was from 60 to 84 mm. of mercury. Similarly in the 20 experiments on decapitate animals, in the 4 which showed vaso-motor reversal with yohimbine the average blood pressure was 60 mm. of mercury, whereas the blood pressure range for the remainder of the group was from 24 to 52 mm. of mercury. In general these results are in agreement with those of Raymond-Hamet (1935) but a point not previously mentioned is that in the spinal cat yohimbine more often than not fails to produce a reversal (*vide supra*). Our experiments indicated that if the level of the blood pressure were low for the type of preparation, yohimbine, at least in this particular dosage, merely diminished or annulled the effect of adrenaline but did not reverse it.

In every case we found the action of yohimbine entirely independent of the anaesthetic used.

A fact not sufficiently stressed in the literature on ergotoxine is that the vaso-motor reversal with this drug is by no means constantly obtained. In contrast, we found yohimbine 100 p.c. effective with the above qualification. An additional advantage that yohimbine possesses over ergotoxine is that since it does not of itself produce a rise in blood pressure, its effect on adrenaline can be obtained without any

initial increase in blood pressure. We therefore agree with Raymond-Hamet (1935) that, as a means of producing vaso-motor reversal, yohimbine is preferable to ergotoxine.

The literature contains slightly conflicting results of the action of yohimbine on the isolated frog's heart. Weger (1935) working with both the frog and the cat found that yohimbine itself had a slight motor effect which changed to a progressive inhibition and that it did not prevent the action of adrenaline on the heart. Chang Shaw Jang (1941) on the other hand, working only on the frog's heart, observed "no effect on the action of adrenaline with yohimbine in concentrations below 1: 10^5, at which, however, yohimbine slightly depressed the heart and markedly diminished but never reversed the adrenaline action".

In concentrations below 1: 10^5 we were able to confirm some results of these workers in that yohimbine had no effect on the action of adrenaline on the heart. However, in higher concentrations of yohimbine we obtained a much more depressing effect than that apparently observed by Jang (1941) for we were unable to evaluate the effects on the adrenaline action in these concentrations.

The perfused frog's blood vessels gave more definite results, but we were able to obtain a marked diminution of the adrenaline effect only if the latter were added to the perfusion fluid within 2 to 3 minutes of the addition of yohimbine. Consequently we are not in complete agreement with Weger (1935) who found that according to the concentration of yohimbine the vaso-constrictor action of adrenaline was weakened and might be changed to a vasodilation.

In the case of the isolated rabbit's uterus, with dilutions ranging from 1 : 10^6 down to 1 : 10^8 we obtained a diminution but not a reversal. In comparison Weger (1935), working with the same preparation but with dilutions ranging from 1 : 10^5 to 1 : 10^6, found that yohimbine either weakened, abolished or inverted the action of adrenaline. That he was able to obtain this marked result was presumably due to the higher concentrations of yohimbine employed.

A final point with which we were in agreement with Weger (1935) was the effect of yohimbine on the isolated rabbit's intestine where he found that the inhibiting action of adrenaline was easily weakened or abolished by moderate doses of yohimbine. Carrying the experiment further we found that stronger concentrations of yohimbine (0·1 cc. of 1 : 10^3) actually abolished the response of the rabbit's intestine to adrenaline.

SUMMARY.

The action of yohimbine on the vaso-motor response to adrenaline has been investigated with anaesthetized, decerebrated and decapitated cats.

The efficiency of yohimbine in producing this vaso-motor reversal is compared with that of ergotoxine.

The action of yohimbine on the response of other tissues to adrenaline has also been investigated.

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